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THE UNIVERSITY OF ALBERTA

MECHANISMS OF INTRACELLULAR ICE FORMATION DURING RAPID COOLING

(C)

by

KENNETH B. MULDREW

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

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FALL, 1988

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FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled **MECHANISMS OF INTRACELLULAR ICE FORMATION DURING RAPID COOLING** submitted by **Kenneth B. Muldrew** in partial fulfilment of the requirements for the degree of **Master of Science** in **Experimental Pathology**.

(Supervisor)

Date: October 5, 1988

ABSTRACT

When cells are frozen at very rapid cooling rates, ice forms in the interior of the cells. This phenomenon will not occur when cooling is slow as the cells remain in osmotic equilibrium with the extracellular unfrozen fraction. Currently, there are three hypotheses which describe intracellular freezing; these theories have been critically analyzed and found to be unable to explain the results of the experiments that were carried out. A new hypothesis is put forward to explain the phenomenon of intracellular ice formation during rapid cooling.

The first theory of intracellular freezing holds that critical supercooling of the protoplasm leads to spontaneous nucleation. It was found that less supercooling was required to cause intracellular freezing as more cryoprotectant was added to the sample. The cells should have formed intracellular ice at the same degree of supercooling regardless of the composition of the bathing solution. The second theory asserted that when the minimum radius of growing ice crystals in the extracellular space matched the radius of aqueous pores in the cell membrane, then these growing crystals would move through the pores and nucleate the protoplasm. It was found that when cells are cooled at a constant rate, the temperature at which intracellular ice formed was independent of the cooling rate. The geometry of ice crystals is strongly dependent on cooling rate thus the theory does not explain the observations. The third hypothesis stipulated that electrical transients at the ice interface could cause the plasma membrane to rupture thereby allowing ice from the extracellular compartment to

nucleate the intracellular compartment. Experiments were performed in which the velocity of the ice interface was measured (the magnitude of electrical transients is proportional to the ice interface velocity) and compared with the degree of intracellular freezing that ocurred under these conditions. The velocity of ice interface which yielded 50% intracellular freezing varied significantly for various concentrations of cryoprotectant.

A hypothesis consistent with the observations is proposed, in which the plasma membrane is ruptured when a critical gradient in osmotic pressure across the membrane is exceeded and the protoplasm is nucleated by extracellular ice.

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LIST OF ABBREVIATIONS

atm	-	Atmospheres
dH2O	-	distilled water
DMSO	-	dimethyl sulfoxide
HEPES	-	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
g	-	acceleration due to gravity
L-glutamine	-	levorotatory glutamine
M	-	molar
MEM	-	minimal essential media
TCM	-	tissue culture media
TSAI	-	tolerable surface area increment
µl	-	microliters
1x	-	one times isotonic concentration
10x	-	ten times isotonic concentration

CHAPTER I

INTRODUCTION

1.1 Life at Low Temperatures

The study of cryobiology is the examination of life at low temperatures; low temperatures being those in which water exists as a solid. Without liquid water, life as we know it does not exist. There is, however, a peculiar phenomenon which can occur when living material is frozen: life can be suspended. That is, life can be resumed when returned to temperatures above 0°C. The biological clocks which control living organisms from birth to death can actually be stopped and then started again at a later physical time as if no real time had passed at all. This is surely one of the most fascinating concepts that can be presented to a life form which is conscious of its own mortality. For who among us does not wonder what the future may bring long after our lives are over. The act of freezing ourselves promises to let us view the future - but there are problems which stand between our present and a future in which we can actually travel to the future (ay, there's the rub). The principal difficulty lies in the fact that to be frozen, you have to get there first; and getting there involves traveling through physical environments which are very hostile to living things. Thus it is not the act of being frozen, but the process of freezing (and thawing) which must be overcome before these promises are realized. Cryobiology is the science devoted to discovering the responses of living things when they are placed in this zone that lies between active life and suspended life. Presently, it is only possible to preserve single cell suspensions and simple tissues at low temperatures. The

inability to successfully freeze more complex tissues and organs stems, in part, from a lack of understanding of the nature of injury which occurs during freezing and thawing.

1.2 Objectives and Tools

The objectives of this thesis are to probe into the events which occur when cell suspensions are cooled rapidly: a procedure which usually results in the formation of ice crystals inside cells. This occurrence is typically related to lethal injury of the cells (it has been hypothesized that a small amount of ice inside cells is not necessarily lethal) rendering the use of rapid cooling unacceptable in protocols which are designed for the low temperature preservation of biological materials. In order to design methods for avoiding injury during freezing, it is necessary to understand how that injury is caused in the first place. This study focuses on the nature of intracellular ice formation: how it forms and what is the damage associated with it. This is not the first time that these questions have been addressed and naturally, theories of the cause of intracellular ice and its related injury do exist. These theories will be addressed in this thesis and tested experimentally. The end result will be a rejection of the present theories and the proposal of a new theory which better conforms to the experimental evidence.

The principal tool which will be used to examine the nature of intracellular freezing will be the cryomicroscope. Although the freezing process has been viewed microscopically for almost two centuries, it has not been until recently that the technology of

cryomicroscopy has evolved into a sophisticated tool which allows controlled experiments to be carried out to study the freezing of living tissues. Recent advances in microelectronics, video and computer technologies have led to the construction of excellent general purpose cryomicroscopes whose potential has yet to be fully tapped.

Until lately, the study of cryobiology was mainly limited to viewing samples before and after freezing while the events which occurred during freezing had to be presumed. Ingenious experiments had to be designed in order to discover what was happening during the freezing process. The exact nature of the events that occurred during freezing itself could only be inferred from what could be observed before or after. Now it is possible to actually watch cells as they are frozen and thawed under a myriad of possible conditions (earlier cryomicroscopes allowed only crude control of the freezing environment). The general purpose nature of these recent developments means that it will be some time before the possibilities are exhausted. This new technology is just beginning to be exploited in our search for the responses of living things to low temperatures and it is proving very useful.

1.3 Cellular Responses to Low Temperatures

The principal point explored by this thesis relates to the cause of intracellular freezing during rapid cooling. When cells are frozen at relatively slow cooling rates, ice first forms outside the cells. Since ice consists of pure water, the solutes that are present in the solution become concentrated in the unfrozen compartment. This presents an

osmotic stress to the cells causing them to shrink in order to maintain osmotic equilibrium. As the temperature drops further, more ice is formed and the solute concentration increases proportionally. If cooling is slow enough that osmotic equilibrium is maintained, then no ice will form inside the cells. They will dehydrate to such an extent that no freezable water is left inside. If cooling is sufficiently rapid, however, ice will form inside the cells. It is this event which will be discussed and a theory developed in which the formation of intracellular ice is a result of the rupturing of the plasma membrane when the osmotic pressure gradient across the membrane reaches a critical level. This allows ice from the extracellular compartment to nucleate the protoplasm. This is a novel theory describing the formation of intracellular ice and it will be shown to be the best one that is currently available.

1.4 Experimental Approach

In order to prove this hypothesis, it is necessary to show that a correlation exists between the magnitude of the osmotic pressure gradient and the incidence of intracellular freezing. The approach that will be taken to show this consists of supercooling cell suspensions (cooling them below their freezing point in the absence of ice) to various degrees on the cryomicroscope and then initiating ice formation artificially. This allows the osmotic pressure gradient to be calculated as the osmotic pressures inside and outside the cells can be calculated. This experiment will also be used to provide evidence against current theories of intracellular ice formation as will an

experiment in which cell suspensions are cooled at a constant rate (with ice formation being initiated before this constant cooling is started, just below the freezing point of the solution). The next step is to show that the plasma membrane is ruptured in cells which have frozen intracellularly but not in cells which have experienced identical conditions without ice forming intracellularly. Then it will be shown that a high osmotic pressure gradient can cause the plasma membrane to rupture in cells which are not exposed to ice (neither intracellularly nor extracellularly). These novel experiments will provide compelling evidence to accept the osmotic pressure gradient theory of intracellular freezing.

CHAPTER II

LITERATURE REVIEW

2.1 Introduction

To begin the thesis, the relevant literature has been reviewed with a focus on intracellular freezing and cryomicroscopy. Although the vast majority of the cryobiology literature deals with approaches and techniques for preserving cells and tissues, the review presented here deals almost exclusively with theoretical aspects of the freezing of living cells and the use of cryomicroscopy in the search for such theories. The information is presented chronologically as this approach best allows the origins and development of cryomicroscopy and cryobiology to be traced and the effects that each had on the other illustrated.

The origins of the modern science of cryobiology lie tightly intertwined with the advent of cryomicroscopy. In the early part of the nineteenth century, German botanists began wondering about the nature of life and death at low temperatures. They first took their microscopes outside in the winter to observe what happened when ice formed in living tissues. From these observations grew the application of the scientific method to the study of life at subzero temperatures.

2.2 German Botanists

In 1830, Goeppert first observed plant cells during freezing by taking his microscope outside during the winter and observing tissues as they cooled to reach the outside temperature. He refuted the view of earlier botanists (Duhamel, Senebier, Fafn) that freezing death is

caused by ice forming and expanding inside cells thereby tearing and rupturing them. Far from this, Goeppert observed ice forming between cells - not inside them - causing the cells to shrink as a result of dehydration. Goeppert was probably among the first people to actually observe ice in a living tissue. The view of earlier botanists was based on observations of the incredible forces that could be generated when water froze (the expansion that accompanies ice formation can easily break large rocks). Goeppert's observations must have surely come as quite a revelation, for they indicated that it might be possible to preserve life by freezing (7).

Sachs, in 1860, put his microscope in an open window when the outside temperature fell to about -5°C or -6°C and also observed plant cells during freezing. He confirmed Goeppert's observations that ice only forms in the interstitium (the spaces between cells) and causes dehydration of the cells. His observations led him to believe that cells die on thawing, not freezing, and that slow thawing might allow cells to survive (28).

Müller-Thurgau, further advancing the techniques of cryomicroscopy, placed his microscope in an ice box containing a salt-water-ice solution to observe freezing in plant cells. He noticed that the tissues needed to be supercooled in order to freeze and that when they could be held below 0°C in the absence of ice no damage resulted upon rewarming. Refuting Sachs' belief, Müller-Thurgau showed that the rate of thawing was of little or no consequence to the survival of most plant cells. He argued that cell death was a result of dehydration caused by the

formation of ice and was able to show that the majority of water contained in tissues had become ice by the time lethal temperatures were reached (23).

At the close of the nineteenth century, Molisch built the first cryomicroscope - a microscope designed specifically for observing specimens during freezing. Since this is a landmark event in cryomicroscopy, it is worth describing Molisch' microscope in some detail. It consisted of an insulated wooden box with a salt-water-ice bath inside. In the center was another box containing the microscope. Panning of the stage and focusing was accomplished by controls which extended to the exterior of the outer box. The entire apparatus was covered with a lid to further insulate it (with an opening for light to enter). A thermometer bulb was positioned beside the sample to monitor temperature and a relatively constant temperature of about -10°C could easily be maintained for several hours in a room temperature of about 5°C. The temperature of the sample could be precisely controlled by varying the composition of the solution in the ice bath (22).

Using this cryomicroscope, Molisch confirmed Müller-Thurgau's finding that with progressively lower temperatures, a greater amount of ice is formed. He also found that leaves which were partially dehydrated before freezing suffered less damage than normal leaves after a freeze-thaw cycle. He proposed that the concentration of solutes in the protoplasm could be toxic or could lead to reactions or precipitates which would be toxic (22).

In the early part of the twentieth century, Wiegand demonstrated that ice could be formed in the interstitium of dormant buds and caused the cells to be almost totally dehydrated. These cells survived freezing and thawing despite the fact that Molisch' theory of chemical toxicity as a result of dehydration predicted that they should have died (38).

2.3 Development of the Cryostage

In 1918, Schander and Schaffnit built the first cryostage, a specially designed microscope stage that allowed observation of freezing events which could be mounted on a normal microscope. Cooling was accomplished by passing compressed carbon dioxide through ether and circulating it through channels in the stage. This apparatus was able to achieve rapid cooling down to about -30°C (29). In 1931 Chamot and Mason improved upon this concept and designed a sophisticated stage for observing chemical phenomena. They circulated a cold liquid through channels in a solid metal block. In the center of the block, a cylinder was removed and a glass slide was placed over the top. The sample was mounted on this glass slide and a thermometer bulb was inserted into the cylinder to monitor temperature. Although they did not implement it, they suggested that more accurate temperature readings could be made by placing a microthermocouple on the glass slide (1).

2.4 Intracellular Freezing

By this time, the study of cryobiology had spread far beyond the domain of the German botanists although it was still primarily the concern of plant physiologists. In 1936, Levitt, studying the

phenomenon of frost hardening (a process by which some plants can change the composition of their cells in order to survive freezing) proposed a mechanism for the formation of intracellular ice. This theory stated that if cooling was rapid enough, a cell could not maintain osmotic equilibrium with the extracellular environment. This would cause the protoplasm to become supercooled and the probability of nucleation would increase. At a certain degree of supercooling, nucleation of the protoplasm would become likely and ice would form inside the cells (10).

At about the same time, Stuckey and Curtis used a modified version of the Mason and Rochow (16) stage to observe plant tissues during freezing. A combination of ether and dry ice was used as the coolant and the objective lens was also cooled with dry ice to prevent it from warming the sample. Nucleation of ice in the sample was accomplished by tapping the cover glass with a dissecting needle that was pre-cooled in dry ice. They were also studying frost hardened tissues and noticed that hardened plant tissue froze intracellularly at much lower temperatures than non-hardened tissue. They were also unable to demonstrate cell death (as a result of freezing) in the absence of intracellular ice formation and even went so far as to say that this effect had never been proven. They concluded that freezing death to cells was solely due to intracellular ice formation (37).

2.5 Early Theories of Freezing Injury

When Luyet reviewed the work that had been done up until this point, he proposed that in order for cells to survive freezing, the protoplasm must vitrify (13). Vitrification is the process of

transforming a liquid into an amorphous glass solid, avoiding the crystalline state altogether. In order to vitrify pure water, small droplets must be cooled extremely rapidly; if solutes are added, however, the process can occur much more readily. At this time, four major theories of freezing injury existed:

1. Chemical effects of dehydration : concentration of cell sap is toxic or toxic precipitates form on dehydration. Müller-Thurgau, Molisch. (23,22)
2. Physical effects of dehydration : Rapid dehydration may lead to tearing of the plasma membrane. Chandler and Hildreth. (2)
3. Mechanical force of ice outside cells : cells are crushed, torn or lanced by the extracellular ice. Iljin. (9)
4. Mechanical force of ice inside cells : Ice destroys the cellular organization and contents. Stuckey and Curtis. (37)

2.6 Low Temperature Preservation

In 1949, one of the most important papers in the history of cryobiology was published. Polge, Smith and Parkes described how the addition of glycerol before freezing allowed bovine spermatozoa to revive after thawing from -79°C (27). This stimulated an enormous amount of interest in the field as the implications were very clear: life could be preserved at low temperatures. This heralded the beginning of a new phase of cryobiology in which the qualitative observation of events during freezing became less important and the accomplishment of controlled experiments with quantitative results became more predominant. Attempts were made to successfully freeze and thaw many different cell types by adding glycerol as well as numerous

other solutes.

2.7 Developments in Cryomicroscopy

The same group that made this discovery continued to advance the state of cryomicroscopy in order to elucidate the mechanism by which glycerol provided its protective action. Their first attempt consisted of a perspex slide which had strips of copper extending from either side. By immersing the copper strips in either liquid air or warm water, they could control the temperature of the stage. A copper-constantan thermocouple was placed beside the sample to monitor temperature and a film of glycerol was spread over the coverslip to avoid fogging (8). This stage was later redesigned with the main body being a piece of brass with a cylinder removed from the center. A glass slide was placed over the cylinder and a small groove was cut in the slide to allow the thermocouple to be placed under the sample. Heating coils were wrapped around the brass to facilitate rapid warming (32). With this equipment they found that avoidance of ice in the extracellular space is not a requirement for freeze thaw survival and that cells treated with glycerol may avoid intracellular freezing by vitrifying due to the glycerol inside the cell (33).

2.8 Lyotropic Effects of Solutes on Red Blood Cells

At about the same time, Lovelock, working with red blood cells, hypothesized mechanisms for both the nature of freezing damage and the protective action of glycerol against freezing damage. He found that during freezing, the cells were exposed to very high concentrations of electrolytes as these solutes were excluded from the ice matrix. He

hypothesized that these electrolytes had a lyotropic effect on the cell membrane and that prolonged exposure would damage the cell membrane and cause lysis upon dilution to isotonic conditions. By exposing cells to similar concentrations of electrolytes in the absence of ice, he found that the cells suffered identical damage as during freezing. He also hypothesized that intracellular ice would cause a high concentration of intracellular electrolytes to act in the same manner (11). Lovelock proposed that the protective action of glycerol was due to its ability to buffer the electrolyte concentration during freezing (12). Due to the colligative properties of glycerol in solution, it reduced the electrolyte concentration at a given temperature.

2.9 Cryomicroscopic Observation of Red Blood Cells

In the late fifties, Luyet and Rapatz designed a simple but effective stage for observing red blood cells during freezing. The cell suspension was placed between two coverslips which were sealed with vaseline. This apparatus was then immersed in a pre-cooled alcohol bath on the microscope stage and the cells were observed as they cooled to the temperature of the alcohol (14). They used this stage to make several observations on the behavior of erythrocytes during freezing. Immersing the cells into alcohol at -20°C caused the cells to rapidly hemolyze on rewarming; they thought that this was due to intracellular freezing but could not confirm it. Immersion of the cells into alcohol at -3°C did not cause lysis upon rewarming despite the fact that they had observed the cells being squeezed through narrow channels in the ice matrix. If, however, the cells immersed at -3°C were then subjected to

a slow cool (-1°C/Min) to -10°C, then the cells would slowly hemolyze during rewarming (15).

2.10 Analytical Model of Freezing Injury

Mazur, in 1963, took a new approach to the problem of freezing injury by constructing an analytical model of the cell's response to freezing. He developed equations to describe the kinetics of water movement out of cells in response to the hyperosmotic unfrozen fraction. He reasoned that it was necessary to keep the cells close to osmotic equilibrium in order to avoid intracellular freezing. Mazur also proposed a new theory of the mechanism by which intracellular ice forms: he felt that at a certain temperature, the radius of advancing ice crystals in the extracellular compartment was small enough to travel through aqueous pores in the cell membrane and nucleate the protoplasm (17).

2.11 Minimum Critical Volume Hypothesis of Freezing Injury

In the late sixties, Meryman demonstrated that non-penetrating solutes other than electrolytes could cause damage to cells during freezing and thawing (21). This was opposed to Lovelock's theory of the lyotropic effects of electrolytes (11). Meryman proposed the minimum critical volume hypothesis which said that a cell can only shrink to a certain minimum volume. If the osmotic stress goes beyond what is necessary to reach that volume, then an osmotic pressure gradient forms across the cell membrane which causes damage to the membrane resulting in lysis upon dilution to isotonic conditions (21).

2.12 Two Factor Hypothesis of Freezing Injury

Following this, Mazur and coworkers proposed the two factor hypothesis of freezing injury. They said that at slow cooling rates, cells were damaged by solution effects: the altered composition of the solutions both inside and outside the cell due to the formation of ice provided an injurious insult to the cell. They did not attempt to explain the nature of damage at the molecular level but merely noted that there was a damage and that it was caused by the altered composition of the solution due to ice formation. At rapid cooling rates, They proposed that cells are damaged by intracellular ice formation and recrystallization. They were able to show that a high proportion of rapidly frozen cells could recover if they were thawed rapidly but not if thawing was slow. From this they inferred that recrystallization of intracellular ice was the event which provided a lethal injury to the cell (18).

2.13 Computer Controlled Cryomicroscopy

In the early seventies, Diller and coworkers applied the rapidly emerging microelectronic technologies to the control of a cryomicroscope stage. Using a computer to control the temperature allowed sophisticated cooling and warming protocols to be used (3). This was a major advance in cryomicroscopy as it allowed a wide variety of experiments to be carried out while also allowing observation of the cells during these experiments. Realizing the tremendous potential of the emerging technology, they also put the computer to work analyzing the images that were recorded using the cryomicroscope. Using digital filtering and pattern recognition techniques, they were able to program

a computer to automatically recognize cells in an ice matrix and measure the volume of the cells (4). By applying this methodology to successive images, the osmotic response of a single cell could be traced throughout the freezing process.

2.14 TSAI Theory of Slow Cooling Injury

In 1978, Steponkus introduced a new theory describing the mechanism of injury that accompanies slow cooling which stipulated that the relative increase in the surface area of the plasma membrane, upon thawing, was the important factor in understanding the mechanism of damage. He postulated that when cells shrink in response to the hyperosmotic extracellular solution, some of the membrane material may actually be lost in the form of vesicles which split off from the membrane. Upon thawing, the dilution stress forces the cells to expand to their previous isotonic volume. Since there is not enough membrane material left to allow expansion to this size, damage to the membrane results. To quantify the response, Steponkus used the tolerable surface area increment, a measure of how much expansion in the cell's surface area could be tolerated before injury occured (36).

2.15 Intracellular Ice as a Consequence of Cell Damage

Using a sophisticated cryomicroscope with phase-contrast optics, Steponkus and Dowgert were able to observe ruptures in the membranes of plant protoplasts immediately before intracellular freezing (5). They proposed that intracellular ice formation was a result of damage to the cell membrane; after rupturing of the plasma membrane, ice from the extracellular compartment was able to move in to nucleate the

supercooled intracellular compartment. Thus intracellular freezing was seen as a result of damage to the cell rather than a cause (34). To explain the mechanism by which the cell membrane might rupture during rapid cooling, Steponkus proposed the theory of freeze-induced electrical transients (35). During freezing in dilute aqueous solutions, large potential differences between the ice and solution can exist due to the Workman-Reynolds effect: the ice phase preferentially excludes one sign of charge creating an electrical potential at the interface. The magnitude of the potential produced depends on the velocity of the ice interface, thus during rapid cooling, it could be large enough to cause an injury to a cell membrane (35).

2.16 Summary of Current Theories of Intracellular Freezing

This brings us to the present time in which there are three major theories to explain the formation of intracellular ice during rapid cooling. The first is Levitt's idea of excessive supercooling of the protoplasm allowing nucleation of the intracellular compartment. This theory proposes that if cooling is sufficiently rapid so that the cell cannot maintain osmotic equilibrium with the extracellular environment, the protoplasm will become supercooled. When this supercooling reaches a critical magnitude, intracellular freezing becomes likely. The second is Mazur's theory of extracellular ice crystals propagating through aqueous pores in the plasma membrane. This hypothesis holds that at a certain temperature, the minimum diameter of advancing ice crystals is smaller than the aqueous pores in the plasma membrane and the ice can move into the intracellular compartment. The third is Steponkus'

hypothesis that intracellular freezing is a result of a rupture in the cell membrane which is caused by electrical transients at the ice interface. These electrical transients can cause the membrane to rupture if they reach a critical magnitude thus allowing ice from the extracellular compartment to nucleate the protoplasm.

CHAPTER III

MATERIALS AND METHODS

3.1 Equipment

3.1 (a) Cryomicroscope Stage

The cryomicroscope stage developed for this study is a convection stage based on the design of Diller et al. (3,30) Refering to figure 3.1, the central foundation consists of a block of plexiglass with a cylinder removed from the center. This material was chosen because of its low coefficient of thermal expansion and the ease with which it can be obtained and worked with. An intake port allows dry nitrogen gas to enter the cylindrical chamber and two exhaust ports allow it to exit after turbulent swirling. There is a glass slide covering both the top and bottom of the cylinder; the sample being placed on the top slide. This slide is cooled by convection from the nitrogen. The bottom of the upper glass slide has a transparent metal oxide coating which is used as a resistance heater by passing an electric current through it (the maximum heating power is ten watts). A 0.001 inch copper constantan thermocouple is mounted on top of the slide in transparent epoxy with a number one cover slip on top of it. The heating is controlled by a proportional controller circuit which is connected to a microcomputer. The computer monitors the temperature by analyzing the voltage from the thermocouple and adds heat as necessary to allow the stage to follow a user programmed thermal protocol. Figure 3.2 shows a photograph of the actual stage used in this study.

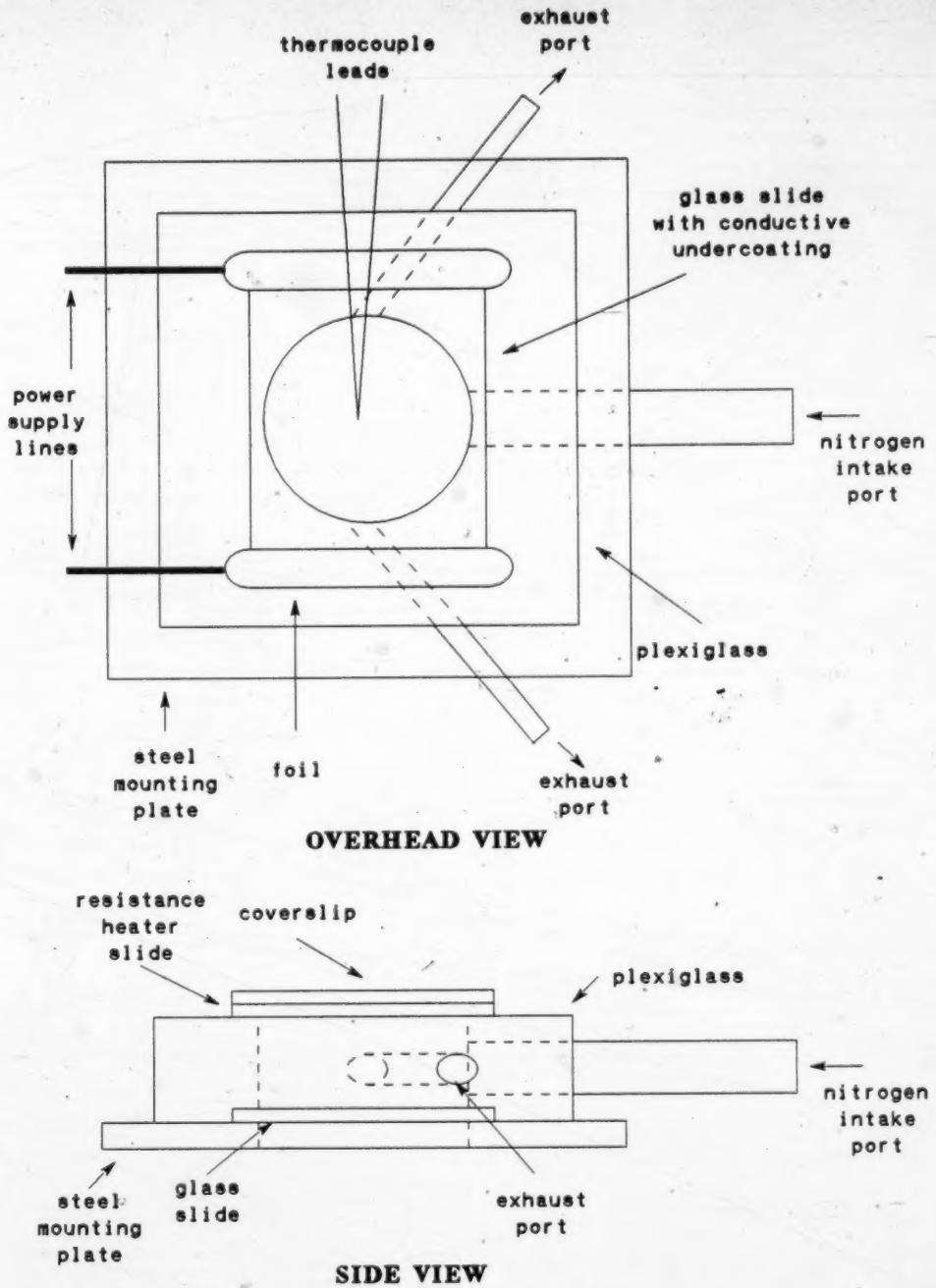

FIGURE 3.1

Schematic diagram of the cryomicroscope stage used in this study. Sample is cooled by convection from nitrogen gas and warmed by passing an electrical current through the metal oxide coating on the resistance heater slide.

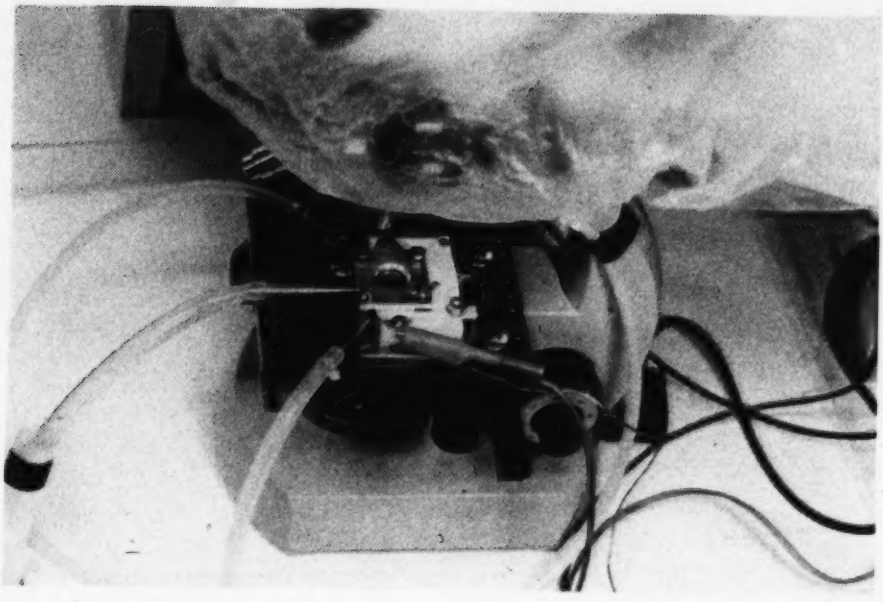

FIGURE 3.2

Photograph of the cryomicroscope stage. The plastic bag covering the upper portion of the microscope is pulled down during operation to trap the dry nitrogen exhaust which prevents fogging of the optics.

3.1 (b) Control Software

The software which controls the stage was developed as a general purpose program which allows any cooling or warming protocol to be specified by the user. The thermal convention is specified in discrete steps: each step consisting of the cooling or warming rate (in °C/min) and the temperature at which to pass control to the next step. As many as thirty steps may be defined for a single protocol. Once the protocol has been entered, the program begins polling the thermocouple 200 times per second. The thermocouple temperature is compared with the ideal temperature (the temperature that the stage should be at in order to follow the prescribed cooling rate) and the appropriate proportional control signal is sent out to either add heat or turn off the heater. This signal consists of a number from zero to fifteen and is proportional to the difference between the measured and the ideal temperatures. The heating signal is also sent 200 times per second. Once started, a protocol may be suspended or changed at any time. Figure 3.3 shows a flow chart of the program which controls the stage illustrating the decision processes involved.

3.1 (c) Cryomicroscope Operation

The operation of the cryomicroscope is reasonably straightforward, however, it still requires a certain amount of skill and practice in order to be reliable and effective. The greatest source of difficulty lies in the control of nitrogen flow to the stage. This is accomplished by a manually operated valve on a nitrogen dewar. The operator is responsible for monitoring the amount of current flowing through the heater, and adjusting the nitrogen flow to maintain a current of about

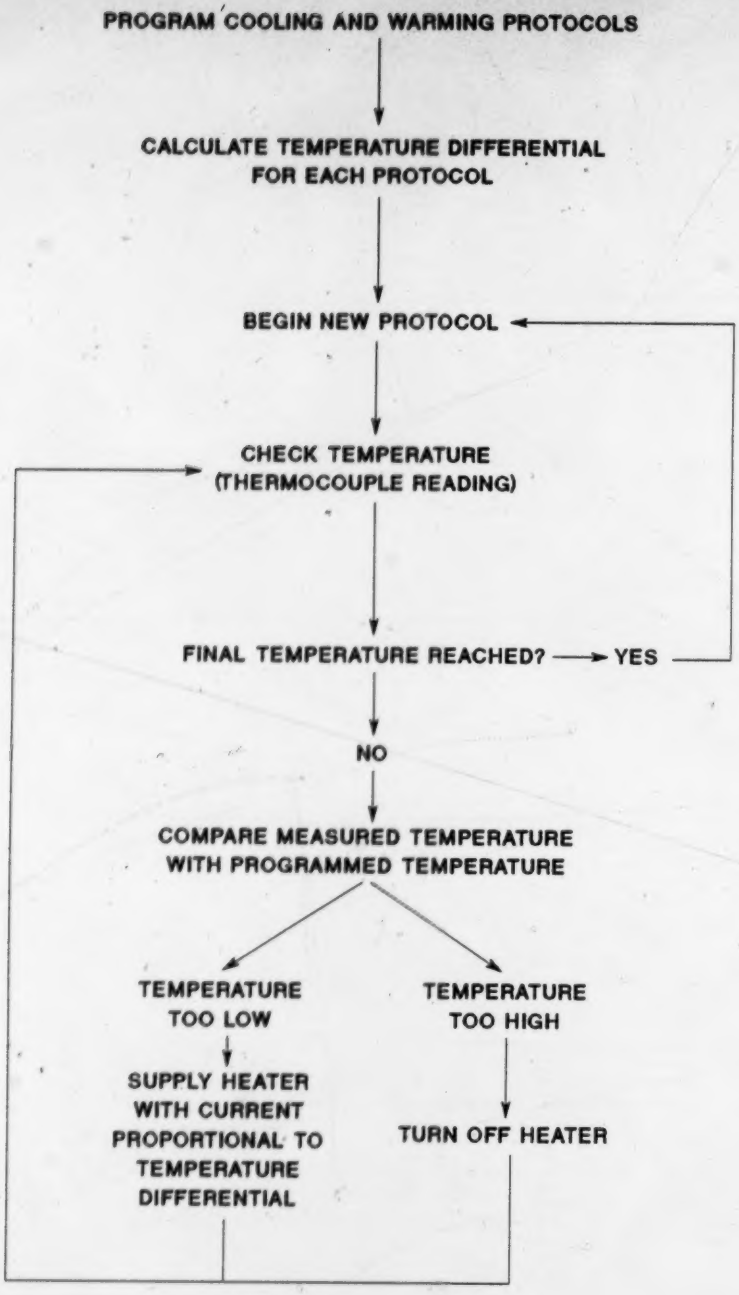

FIGURE 3.3

Flow diagram of the computer program that controls the cryomicroscope stage. The temperature of the sample is checked and the appropriate action taken at a rate of 200 times per second.

100 milliamps. This allows sufficient headroom to keep the temperature accurate (the current must be between 10 milliamps and 200 milliamps to ensure linear tracking). The reason for this manual control is due to the low power of the heater which is a consequence of the high resistance in the metal oxide coating on the glass slide. After a certain amount of practice, an average person can comfortably operate the stage at cooling rates of up to -200°C/min. Cooling at this rate can only be followed to about -50°C although slower rates allow the stage to be taken down to -150°C. The maximum warming rate is about 500°C/min. These limitations are inherent in the design and are primarily due to the high resistance of the metal oxide coating. Although these limits could be a severe handicap when working with certain types of cells, they did not hamper these experiments as they were instrumental in determining the cell type which was used.

To observe cells on the stage, a two micro liter sample is placed on top of the glass slide and a cover slip is applied. This provides a thin film which minimizes thermal gradients and limits the movement of cells. Due to the expansive properties of ice, a sample volume any greater than two µl can lead to the movement of cells before or after freezing. The field of view must be kept within 0.5 mm of the thermocouple junction to ensure that the temperature of the cells being observed is consistent with the programmed temperature. The events are recorded on video tape with a time-date generator overlapping the time onto the video signal. The time at which a protocol is started is recorded so that the time on the video can be used later to determine

the temperature at any given location on the tape. To initiate ice formation, a copper wire (pre-cooled in liquid nitrogen) is held just above the edge of the coverslip, away from the field of view so that it does not affect the temperature of the observed sample. To counter frost build-up on top of the coverslip, the entire stage is enclosed in a plastic bag. The dry nitrogen exhaust from the stage purges the area of moisture and reduces fog and frost that can accumulate at low temperatures. All observations made in this study were under 132x magnification using a 40x objective lens and a 3.3x ocular lens.

3.2 Biological Materials

All experiments used EmT6 mouse fibroblast cells grown in tissue culture exclusively. These cells were chosen primarily because they responded well within the limits of the cryomicroscope. Intracellular freezing occured at cooling rates less than -200°C/min allowing the phenomenon to be studied with the existing equipment. These cells were also readily available and easily maintained. The cells were grown in monolayer in minimal essential media with 25mM HEPES buffer and Hank's salts; this stock solution was modified to also contain 5% fetal calf serum, 0.4% sodium bicarbonate, 100 μg/ml penicillin/streptomycin and 2mM L-glutamine. The cells were harvested every three days by trypsinization and transferred to fresh media. Cultures of these cells last only for about four months, after which morphological changes in the cells become apparent: the culture becomes over-run with small cells with altered permeability parameters. A stock of frozen cells was used to create new cultures every four months although, it should be noted that each new culture had slightly different permeability properties

despite the fact that they were thawed from the same stock. Because of this, no single experiment was carried out using cells from more than one culture. Cell suspensions collected after trypsinization were immediately placed on a shaker in a 4°C refrigerator. The cells were used within four hours of collection; any remaining cells were discarded.

3.3 Solutions:

In the experiments, it was necessary to expose cell suspensions to various concentrations of dimethyl sulfoxide (DMSO) and glycerol. Cells had to be placed in solutions with DMSO concentrations of 0.5M, 1M, 2M, 3M, 4M and 5M and glycerol concentrations of 0.5M, 1M and 2M, however, the concentration of other solutes had to be constant (1x or isotonic concentration) in each suspension. To accomplish this, the following solutions were prepared which could be added to cells suspended in TCM in certain proportions:

4M DMSO in 1x MEM:

28.54 ml DMSO
+ 10.00 ml 10x MEM
+ 61.46 ml dH2O
100.00 ml

8M DMSO in 1x MEM:

28.54 ml DMSO
+ 5.00 ml 10x MEM
+ 16.46 ml dH2O
100.00 ml

4M glycerol in 1x MEM:

29.28 ml glycerol
+ 10.00 ml 10x MEM
+ 60.72 ml dH2O
100.00 ml

For certain experiments, the kinetics of addition of these solutions was critical, while for others, it was only necessary to bring the solutions up to a certain concentration without incurring any damage to the cells as a result of the addition of DMSO or glycerol. For the experiments in which the latter method was used, the following protocols of addition of the above solutions was employed (in each case, the DMSO or glycerol solutions were added to the cell suspensions):

0.5M DMSO - 700 µl of cell suspension was combined with 100 µl of 4M DMSO/1xMEM

1M DMSO - 600 µl of cell suspension was combined with two 100 µl aliquots of 4M DMSO/1xMEM added one minute apart

2M DMSO - 400 µl of cell suspension was combined with four 100 µl aliquots of 4M DMSO/1xMEM added one minute apart

0.5M glycerol - 700 µl of cell suspension was combined with 100 µl of 4M glycerol/1xMEM

1M glycerol - 600 µl of cell suspension was combined with two 100 µl aliquots of 4M glycerol/1xMEM added five minutes apart

2M glycerol - 400 µl of cell suspension was combined with four 100 µl aliquots of 4M glycerol/1xMEM added five minutes apart

To achieve the solutions in which the kinetics of addition were of critical importance, the following protocols were used (again, in each case, the DMSO solutions were added to the cell suspensions):

Rapid Addition of DMSO:

3M DMSO - 600 µl of cell suspension was combined with 360 µl of 8M DMSO/1xMEM

4M DMSO - 480 µl of cell suspension was combined with 480 µl of 8M DMSO/1xMEM

5M DMSO - 360 µl of cell suspension was combined with 600 µl of 8M DMSO/1xMEM

Slow Addition of DMSO:

3M DMSO - 600 µl of cell suspension was combined with six 50 µl aliquots and one 60 µl aliquot of 8M DMSO/1xMEM added 30 seconds apart

4M DMSO - 480 µl of cell suspension was combined with nine 50 µl aliquots and one 30 µl aliquot of 8M DMSO/1xMEM added 30 seconds apart

5M DMSO - 360 µl of cell suspension was combined with twelve 50 µl aliquots of 8M DMSO/1xMEM added 30 seconds apart

Each cell suspension from both groups was allowed to sit for two minutes after the final concentration had been reached. At this time all solutions were diluted with tissue culture media; the kinetics of dilution were identical for each solution and followed the subsequent protocol:

1. six aliquots of 50 µl of TCM were added at 30 second intervals
2. six aliquots of 100 µl of TCM were added at 30 second intervals
3. four aliquots of 300 µl of TCM were added at 30 second intervals
4. four aliquots of 500 µl of TCM were added at 30 second intervals
5. two aliquots of 1 ml of TCM were added at 30 second intervals

This brought the final concentrations of the solutions to between 0.7M DMSO and 0.4M DMSO (5M DMSO resulted in a final concentration of 0.7M DMSO, 3M resulted in 0.4M DMSO). Since the kinetics of addition was the dependent variable in the study, it was felt that it was more important that each group be subjected to identical kinetics in the dilution phase rather than having each group end up at the exact same concentration of DMSO.

3.4 Assays

The determination of the formation of intracellular ice was made by the observation of a "flashing" event. This refers to the phenomenon of instant darkening of a cell when ice forms in the protoplasm and is caused by the scattering of light by the intracellular ice crystals. This distinctive event is very obvious and easy to observe. If a cell is significantly dehydrated when intracellular nucleation occurs, then the darkening can be less pronounced and proceeds more slowly through the cell interior. This has been called "twitching" by Körber et al and they have hypothesized that it may be a different event than "flashing". (31) In all the observations of this study, however, twitching was only observed in cells which were very dehydrated and it was assumed that the ice formed slowly as a result of there being very little water left in the cell which was available for ice formation (water that exists in

conjunction with macromolecules such as the water of hydration of protiens can not form ice). Thus, any darkening of the cell interior was assumed to be due to the formation of intracellular ice without discrimination. Figure 3.4 shows a photograph of a cell before and after intracellular ice formation. The change from light to dark occurs almost instantly.

As an assay for membrane integrity, the ability of the cell to provide a barrier to ice was used. This provides an immediate assay of the integrity of the membrane, which is accurate and easy to perform. Although a damaged cell may still provide a barrier to ice, loss of the barrier properties to ice is clearly indicative of damage to the plasma membrane. In this assay, cell suspensions were cooled on the cryomicroscope to just below the freezing point of the solution and ice formation was initiated. Intact cells provided an obvious barrier to the ice and shrunk osmotically. Damaged cells, however, allowed ice to pass directly into the protoplasm. This is easily observed and the failure of the cell to respond osmotically demonstrates that the observation is not an artifact due to ice moving above or below the cell. Freshly isolated cells were never observed to allow ice to pass through their membrane. Figure 3.5 shows a photograph of a normal cell which provides a barrier to ice and a damaged cell which allows ice to move into the protoplasm.

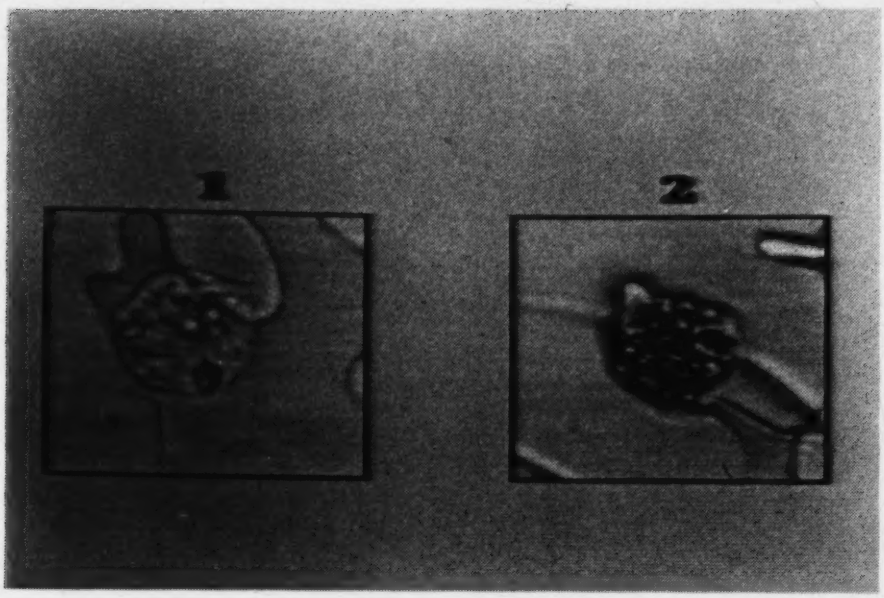

FIGURE 3.4

Photo 1 shows a mouse fibroblast cell in an ice matrix which is being held just below the freezing point. Photo 2 shows the same cell after cooling at -150°C/min to -12°C. The cell interior is dark due to the presence of intracellular ice. Since the protoplasm was supercooled when this ice formed, the crystals are very small. This causes light to scatter rather than pass through the cell freely.

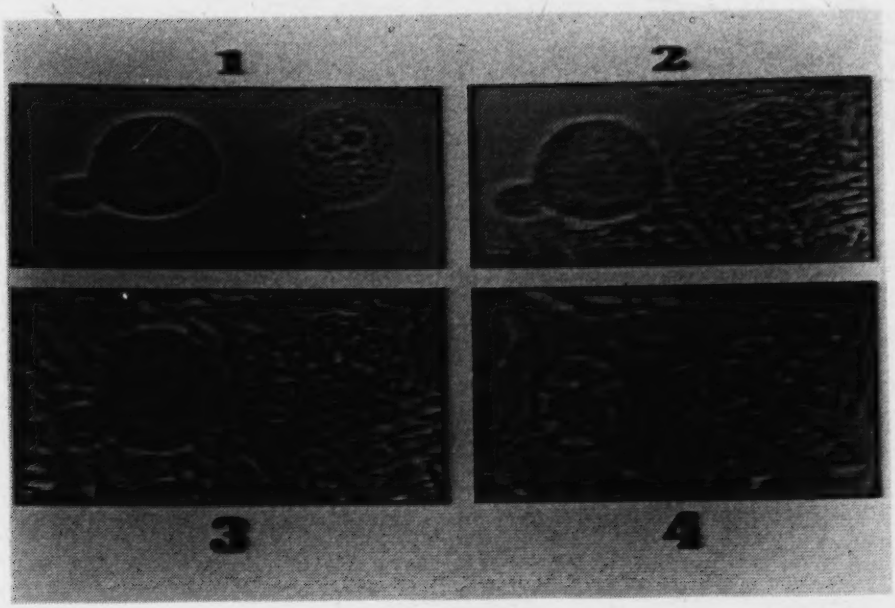

FIGURE 3.5

A series of photographs showing the response of two cells as ice moves through the field of view. The cell on the left provides a barrier to the advancing ice front and shrinks osmotically. The cell on the right, however, allows ice to pass into the protoplasm and no osmotic shrinkage is evident. The frames are numbered in chronological order.

CHAPTER IV

INTRACELLULAR FREEZING AT CONSTANT COOLING RATES

4.1 Introduction

The first set of experiments carried out was to determine the relationships between the cooling rate and intracellular freezing. Cells in tissue culture media and in three concentrations of DMSO were cooled at five different cooling rates on the cryomicroscope and for each case, the percentage of cells in which intracellular ice formed and the temperature at which it formed (if it indeed did form) were recorded. The detailed protocol for the experiment is as follows.

4.2 Experimental Protocol

The sample was cooled to just below the freezing point of the suspending solution, held at that temperature, and the sample nucleated. Table 4.1 shows the temperatures at which this was done for each solution. After ice nucleation, the temperature was held constant for a further 30 seconds to allow the cells to achieve osmotic equilibrium with the unfrozen solution. No further shrinkage was observed after 15 seconds thus the cells were assumed to be completely equilibrated after 30 seconds. The stage was then cooled at one of five pre-determined cooling rates (-70°C/Min, -100°C/Min, -125°C/Min, -150°C/Min or -200°C/Min) to -40°C. The sample was then warmed at +100°C/Min to room temperature. This procedure is depicted more clearly in figure 4.1. The whole procedure was videotaped and the video was later analyzed to determine the proportion of cells in which intracellular ice was observed. Since the cooling rate, the starting temperature and the

TEMPERATURE OF ICE NUCLEATION IN DMSO SOLUTIONS

SOLUTION	TEMP.
1x MEM	-1°C
0.5M DMSO/1x MEM	-3°C
1M DMSO/1x MEM	-5°C
2M DMSO/1x MEM	-7°C

TABLE 4.1

Table showing the temperatures at which ice was nucleated in four concentrations of DMSO.

TIME TEMPERATURE CURVE SHOWING THERMAL PROTOCOL

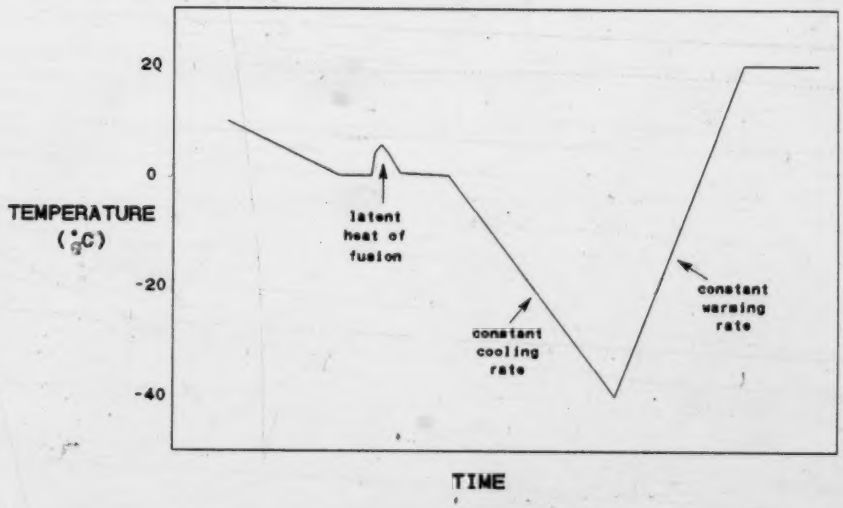

FIGURE 4.1

Graph showing an example of a constant cooling thermal protocol as used in the constant cooling rate experiments.

exact time at which cooling was initiated were known, the temperature at which intracellular freezing occurred could be determined. The time of intracellular freezing could only be determined to the nearest second since that was the accuracy of the time-date generator that was employed. This creates an uncertainty of temperature calculation by a maximum of +/-1.6°C. By analyzing many cells, this source of error was reduced considerably.

4.3 Results

Table 4.2 shows the original data for each of the groups tested. The error for the temperature at which flashing occurred is the standard error calculated from all the samples in each group. The number of cells tested in each group varies slightly because it is impossible to control the number of cells in the field of view each time a protocol is run.

Figure 4.2 shows a graphical depiction of the percentage of cells which freeze intracellularly in each solution at a given cooling rate. In all four solutions, the proportion of cells forming intracellular ice is strongly dependent on cooling rate with faster cooling rates yielding a higher percentage of intracellular freezing. The relationship between the composition of the solution and the incidence of intracellular freezing is less clear. 0.5M DMSO seems to provide the most protection from intracellular freezing and 2M DMSO, the least. The differences in the curves are quite significant, however, the relationship is not immediately apparent.

Figure 4.3 shows a graph of the temperature at which intracellular

INTRACELLULAR FREEZING AS A FUNCTION OF COOLING RATE AND DMSO CONCENTRATION

COOLING RATE (°C/Min)	0M DMSO			0.5M DMSO			1M DMSO			2M DMSO		
	TOTAL CELLS	TOTAL FLASH	TEMP OF FLASH	TOTAL CELLS	TOTAL FLASH	TEMP OF FLASH	TOTAL CELLS	TOTAL FLASH	TEMP OF FLASH	TOTAL CELLS	TOTAL FLASH	TEMP OF FLASH
70	55	5	-8.9±1.6	58	2	-18.8±1.2	55	3	-26.0±1.7	55	6	-29.3±1.9
100	49	8	-13.5±1.4	52	5	-20.3±2.6	44	5	-21.5±3.6	59	22	-28.6±1.2
125	54	17	-11.1±1.3	58	3	-17.4±3.6	52	16	-24.7±1.4	54	24	-29.3±1.2
150	55	20	-13.8±1.0	75	16	-18.9±1.9	62	17	-25.1±0.7	51	35	-30.7±1.0
200	51	33	-9.2±0.5	52	25	-17.7±1.3	57	37	-25.3±1.1	51	48	-26.1±0.9

TABLE 4.2

The table depicts the responses of fibroblast cells to freezing at a constant cooling rate. The proportion of cells which freeze intracellularly as well as the temperature at which this event occurs are recorded. The error in temperature is the standard error of the sample. The term "flash" is used to represent the formation of intracellular ice. Temperature is in °C.

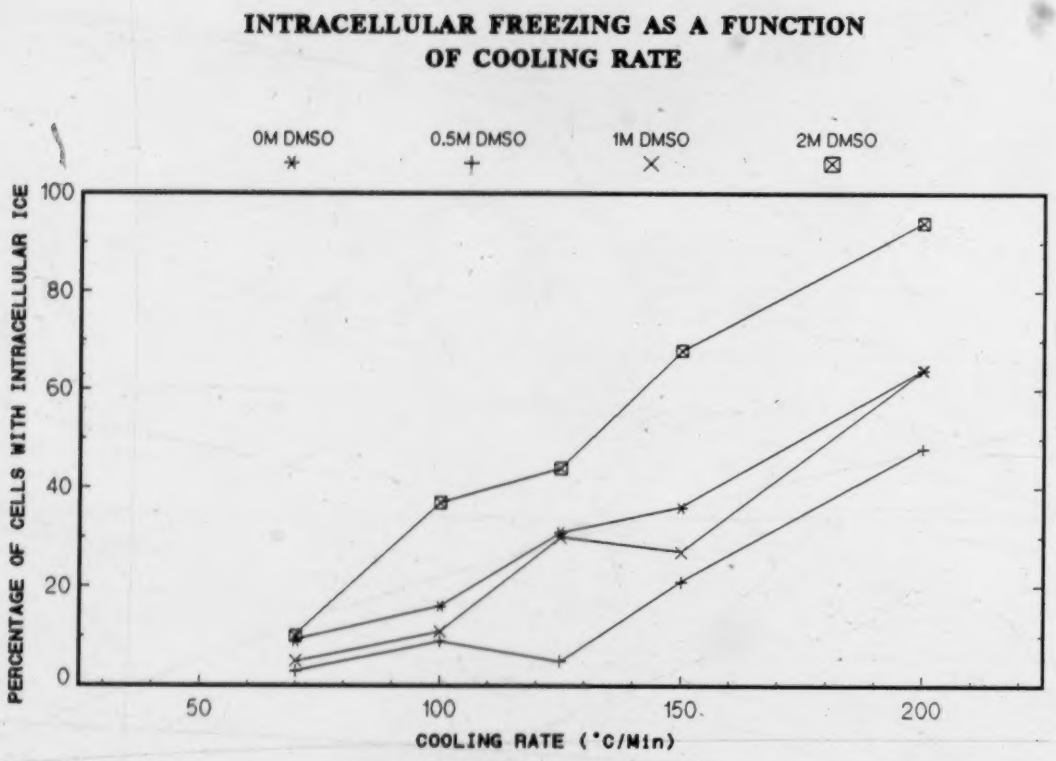

FIGURE 4.2

Graph showing the proportion of cells which freeze intracellularly as a function of cooling rate in four concentrations of DMSO.

TEMPERATURE OF INTRACELLULAR FREEZING AS A FUNCTION OF COOLING RATE

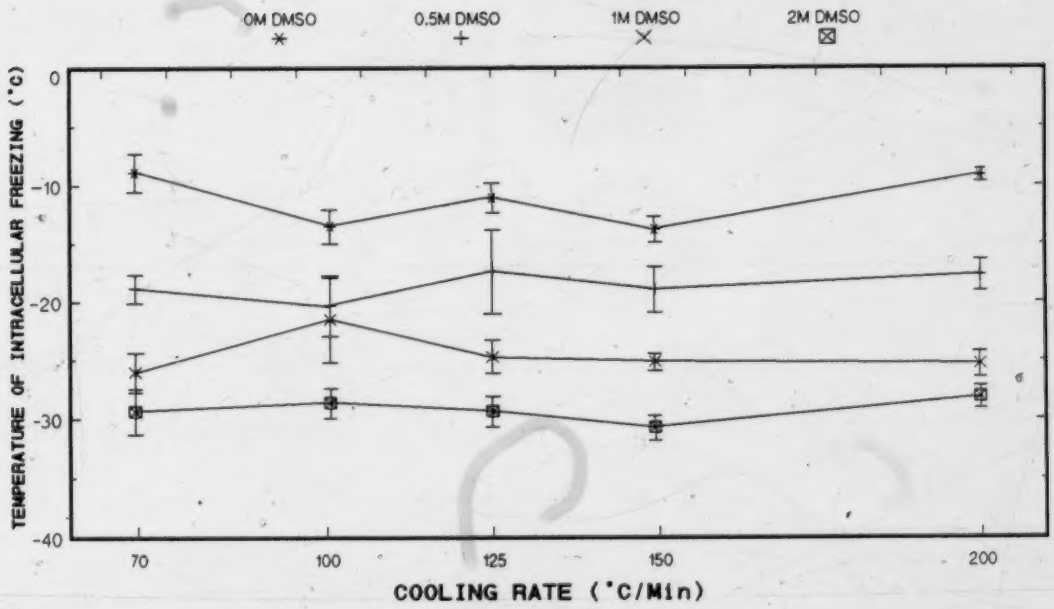

FIGURE 4.3

Graph showing the temperature at which intracellular freezing occurs (if it occurs) as a function of cooling rate in four concentrations of DMSO.

ice forms (if it forms) as a function of the cooling rate for all four solutions. For each solution, it is clear that the temperature at which intracellular ice forms is independent of the cooling rate. It is, however, strongly dependent on the composition of the solution. Clearly, the temperature at which intracellular ice forms decreases as the concentration of DMSO is increased.

4.4 Discussion

Adding a solute to a solvent has the effect of lowering the freezing point of the resulting solution below that of the pure solvent. Assuming an ideal dilute solution, the degree to which the freezing point is depressed is proportional to the molar concentration of solute. This is why the four suspending solutions used in these experiments were nucleated at different temperatures. The amount of ice present at any given temperature is also dependent on the concentration of solute. There are properties of the extracellular environment at subzero temperatures which are strongly dependent on the cooling rate that is being followed as well. The velocity of the advancing ice interface and the morphology of the newly forming ice crystals at this interface are two such properties.

In these experiments, intracellular freezing was seen to occur at the same temperature regardless of the cooling rate within a given solution. Thus the mechanism by which intracellular ice forms must be independent of cooling rate within the limits of this experiment and consequently, not a function of properties of the extracellular environment which are dependent on the cooling rate.

Mazur's theory of intracellular freezing states that when conditions are such that the radii of advancing ice crystals are less than the radii of aqueous pores in the plasma membrane, the crystals will propagate through the membrane and nucleate the protoplasm. The protoplasm will be supercooled if cooling is sufficiently rapid and intracellular freezing will result. The observations presented here show that within a given suspending solution, intracellular freezing occurs at the same temperature regardless of the cooling rate that was followed to reach that temperature. In order for Mazur's theory to be correct, the size of the advancing ice crystals must be independent of cooling rate. In fact, the size of ice crystals forming during cooling at -200°C/min (at a given temperature) will be much smaller than those forming during cooling at -70°C/min (at the same temperature) in the same suspending solutions as this property is strongly dependent on cooling rate.

Steponkus' theory of intracellular freezing holds that electrical transients at the ice interface cause the plasma membrane to rupture thus allowing extracellular ice to nucleate the intracellular compartment. The rupture occurs when the potential difference at the ice interface exceeds a critical level. Since the potential difference is proportional to the velocity of the advancing ice interface (35), intracellular freezing should occur when conditions are such that the ice interface velocity reaches a critical level. For this theory to be consistent with the experimental evidence, it is necessary to assume that the velocity of the ice interface is independent of cooling rate.

This is simply not true; the ice interface velocity is strongly dependent on the cooling rate thus the theory is not supported.

CHAPTER V

INTRACELLULAR ICE FORMATION AFTER NUCLEATION AT SUPERCOOLED TEMPERATURES

5.1 Introduction

This series of experiments was designed to observe intracellular ice formation when the ice in the extracellular compartment was nucleated only after a certain degree of supercooling had taken place. The cell suspension, in tissue culture medium with 0M, 0.5M, 1M or 2M DMSO or the same concentration of glycerol, was supercooled to a predetermined temperature and held at that temperature while ice formation was initiated. These experiments were designed to reveal several aspects of intracellular ice formation in a simple and reliable manner. By using a single step change from unfrozen to frozen at a prescribed temperature, quantitative descriptions of the environment could be realized without resorting to differential equations which become necessary when kinetics are of primary importance. (19,20) The velocity of the advancing ice interface could be measured directly as it moved through the field of view, the degree of supercooling was known and the magnitude of the osmotic pressure gradient across the plasma membrane could be calculated. For the latter, one important assumption was made in the interpretation of this experiment: when ice formation was initiated at a supercooled temperature, the stage would have enough cooling power to buffer the latent heat of fusion produced by ice formation. If the stage could not buffer the latent heat of fusion, the temperature of the sample would rise and the conditions would not be accurately known. It was experimentally determined that the temperature

of the stage rose by less than 1°C and returned to the set temperature within less than half of a second. The assumption was therefore accepted as reasonable.

5.2 Experimental Protocol

A sample of cells in one of the seven different solutions was placed on the stage and cooled at -20°C/min to a predetermined temperature between -3°C and -16°C in the absence of ice. Ice formation was initiated and the temperature was held constant for 15 seconds. The stage was then warmed at +100°C/min to room temperature. The proportion of cells which froze intracellularly and the velocity of the ice interface were determined experimentally for each condition. To measure the ice interface velocity, distance on the viewing screen was calibrated with a hemocytometer and the distance over which the ice traveled in one video frame (1/30 second) was measured. Two different cultures of cells were used for this experiment, one for the DMSO work and one for glycerol.

5.3 Results

To make the interpretation of this set of experiments easier, the measured values of ice interface velocity, degree of supercooling and osmotic pressure gradient have all been mapped graphically as functions of the temperature at which ice nucleation occured. Overlayed on these graphs, are the points at which 50% intracellular freezing occured. These points are found by finding the temperature on the abscissa at which 50% intracellular freezing occured, for a given solution, and moving vertically to the point at which the plotted data is encountered.

This intersection is marked as the point of 50% intracellular freezing and is used to indicate the degree of ice interface velocity, degree of supercooling or osmotic pressure gradient that correlates with 50% intracellular freezing.

Table 5.1 shows the proportion of cells forming intracellular ice in the DMSO experiments and Table 5.2 shows the similar results for the glycerol experiments. Many cells were examined for each case to build confidence in the results. In figure 5.1, the data from table 5.1 is plotted graphically. The curves were fit using the Bezier method (24) which was chosen because no mathematical function is assumed for the data. Since the purpose of the experiment was to determine the nature of this relationship, it would not be appropriate to assume any type of function which could bias the interpretation. The temperatures at which 50% intracellular freezing occurs are marked as these will be used in later analyses. It is clear, from the glycerol data plotted in figure 5.2, that the two different solutes yield qualitatively similar responses. The difference between the two cell cultures used for the two experiments becomes clear when comparing the curve for 0M DMSO from figure 5.1 with the curve for 0M glycerol from figure 5.2. If the properties of the two cell cultures was identical, then these two curves would also be identical as the conditions are exactly the same. The responses of the cells from each culture in this experiment are clearly different; this precludes any direct comparison between the two.

Figures 5.3 and 5.4 show the relationships between the velocity of the ice interface for the various solutions and the temperature of ice

nucleation in the presence of DMSO and glycerol, respectively. The circles on these curves mark points at which 50% intracellular freezing occurred and a line representing the linear regression fit is marked. On both graphs, the regression line has a negative slope although the line on the glycerol graph is much steeper than that for DMSO.

Figures 5.5 and 5.6 show the relationship between the degree of supercooling and the temperature of ice nucleation. The lines are all parallel at 45 degrees as both axes are essentially measuring the same colligative property. The point of these diagrams is to allow a mapping of the temperatures for 50% intracellular freezing onto a graph of supercooling. The melting points for the respective solutions have been calculated using equations published by Pegg and Fahy (see appendix A). (25,26,6)

These same equations can also be used to determine the composition of these solutions at any given temperature, allowing calculation of the osmotic pressure gradient across the cell membrane on nucleation of ice at each temperature. An assumption is made that the cell was in osmotic equilibrium before nucleation, so the osmotic pressure inside the cell just after ice nucleation is equal to the osmotic pressure of the surrounding media just before ice nucleation. The results of these calculations are plotted in figures 5.7 and 5.8. The points at which 50% intracellular freezing occurs are mapped onto both graphs and the regression fit gives lines with negative slopes. The slopes of both graphs are about equal although the y-intercepts are different. This difference can be attributed to the differences between the two cell

INTRACELLULAR FREEZING UPON ICE NUCLEATION AT SUPERCOOLED TEMPERATURES

TEMPERATURE OF ICE NUCLEATION (°C)	0M DMSO		0.5M DMSO		1M DMSO		2M DMSO	
	TOTAL CELLS	No. OF FLASHES	TOTAL CELLS	No. OF FLASHES	TOTAL CELLS	No. OF FLASHES	TOTAL CELLS	No. OF FLASHES
-3	34	2						
-4	33	4	43	0				
-5	52	16	42	8				
-6	26	21	39	7	40	8		
-7	36	35	52	17	36	4		
-8			48	33	35	12		
-9			36	36	31	9	19	0
-10					39	29	40	5
-11					39	38	39	14
-12							29	12
-13							16	7
-14							16	14
-15							4	4

TABLE 5.1

Data showing the proportion of cells which freeze intracellularly when ice formation is initiated at supercooled temperatures in four concentrations of DMSO

INTRACELLULAR FREEZING UPON ICE NUCLEATION AT SUPERCOOLED TEMPERATURES

TEMPERATURE OF ICE NUCLEATION (°C)	0M GLYCEROL		0.5M GLYCEROL		1M GLYCEROL		2M GLYCEROL	
	TOTAL CELLS	No. OF FLASHES	TOTAL CELLS	No. OF FLASHES	TOTAL CELLS	No. OF FLASHES	TOTAL CELLS	No. OF FLASHES
-3	45	0						
-4	49	4						
-5	51	7						
-6	48	20	49	0				
-7	42	26	47	3				
-8	48	45	49	6	48	0		
-9			49	23	41	6		
-10			51	25	48	16	48	0
-11			42	33	48	15	43	1
-12					44	30	42	2
-13					48	46	51	8
-14							41	18
-15							46	23
-16							45	36

TABLE 5.2

Data showing the proportion of cells which freeze intracellularly when ice formation is initiated at supercooled temperatures in four concentrations of glycerol.

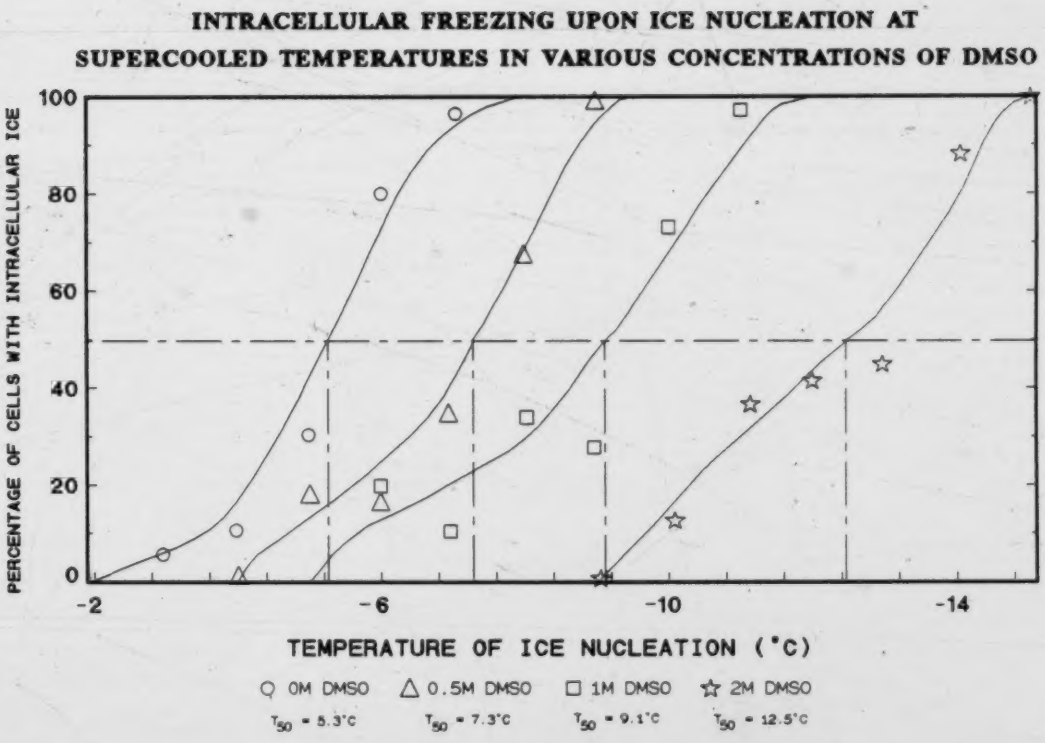

FIGURE 5.1

Graph showing the relationship between intracellular freezing and the temperature of ice nucleation in four concentrations of DMSO. The solid lines are Bezier curves fit to the data points. The temperatures at which 50% intracellular freezing occurs are marked.

INTRACELLULAR FREEZING UPON ICE NUCLEATION AT SUPERCOOLED TEMPERATURES IN VARIOUS CONCENTRATIONS OF GLYCEROL

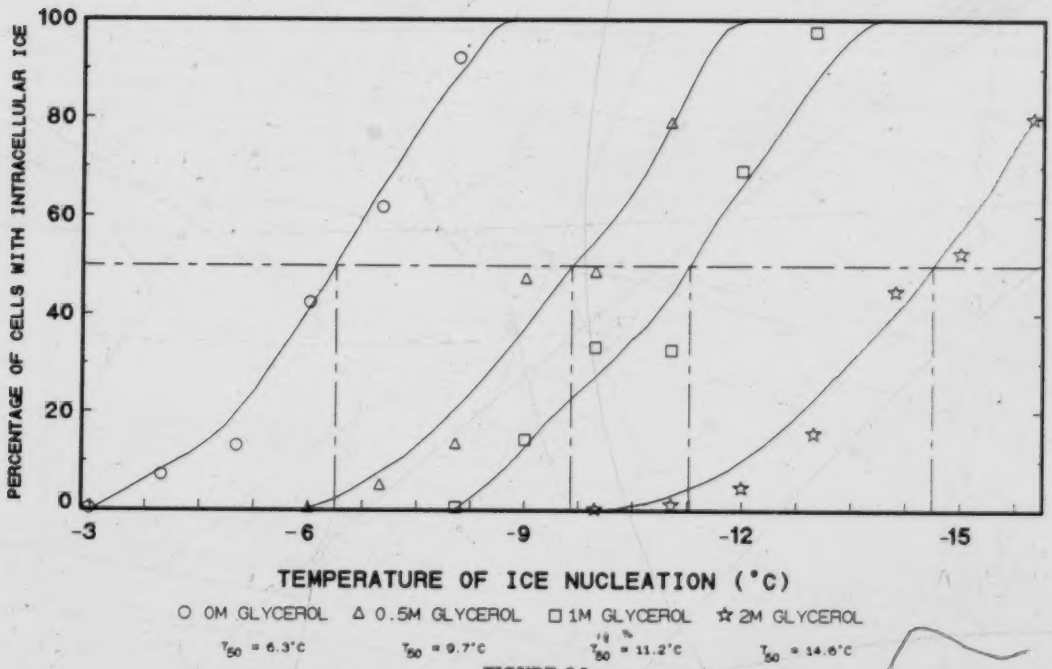

FIGURE 5.2

Graph showing the relationship between intracellular freezing and the temperature of ice nucleation in four concentrations of glycerol. The solid lines are Bezier curves fit to the data points. The temperatures at which 50% intracellular freezing occurs are marked.

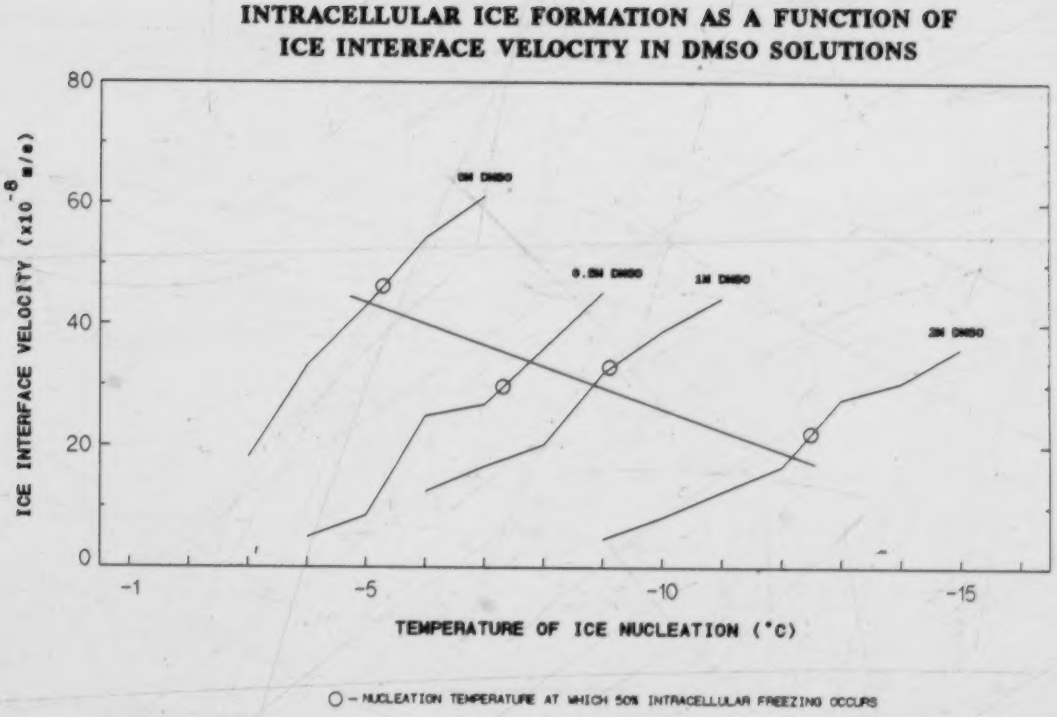

FIGURE 5.3

Graph showing the velocity of the ice interface, upon nucleating at supercooled temperatures, in four different concentrations of DMSO. The temperatures at which 50% intracellular ice forms in the four solutions are mapped on to the respective curves and a redgression line is fitted for these four points.

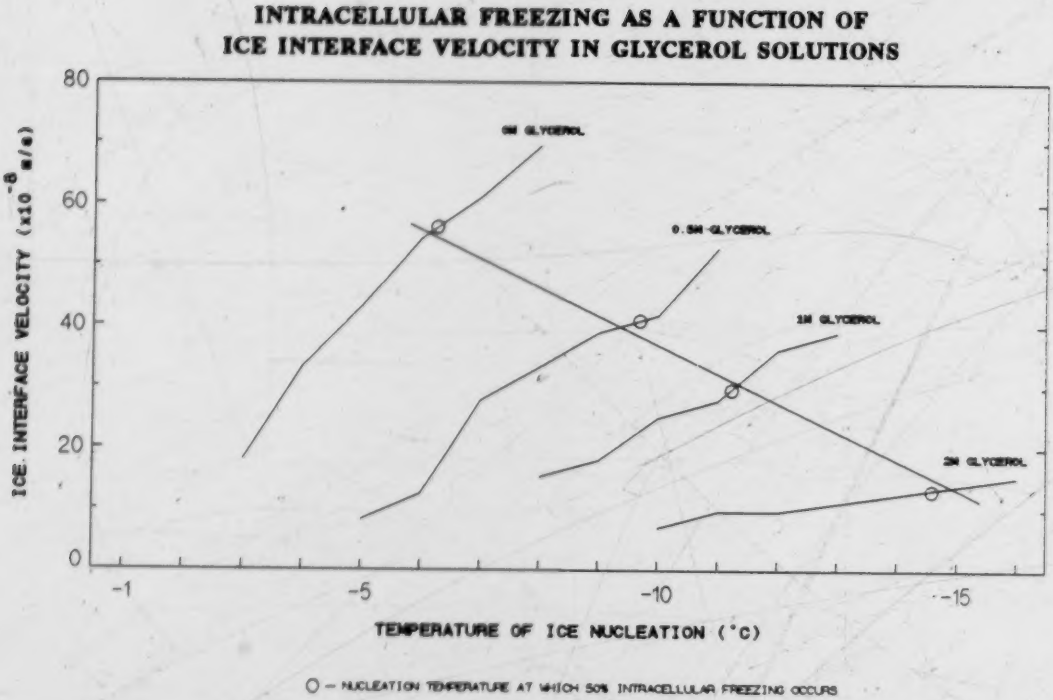

FIGURE 5.4

Graph showing the velocity of the ice interface, upon nucleating at supercooled temperatures, in four different concentrations of glycerol. The temperatures at which 50% intracellular ice forms in the four solutions are mapped on to the respective curves and a regression line is fitted for these four points.

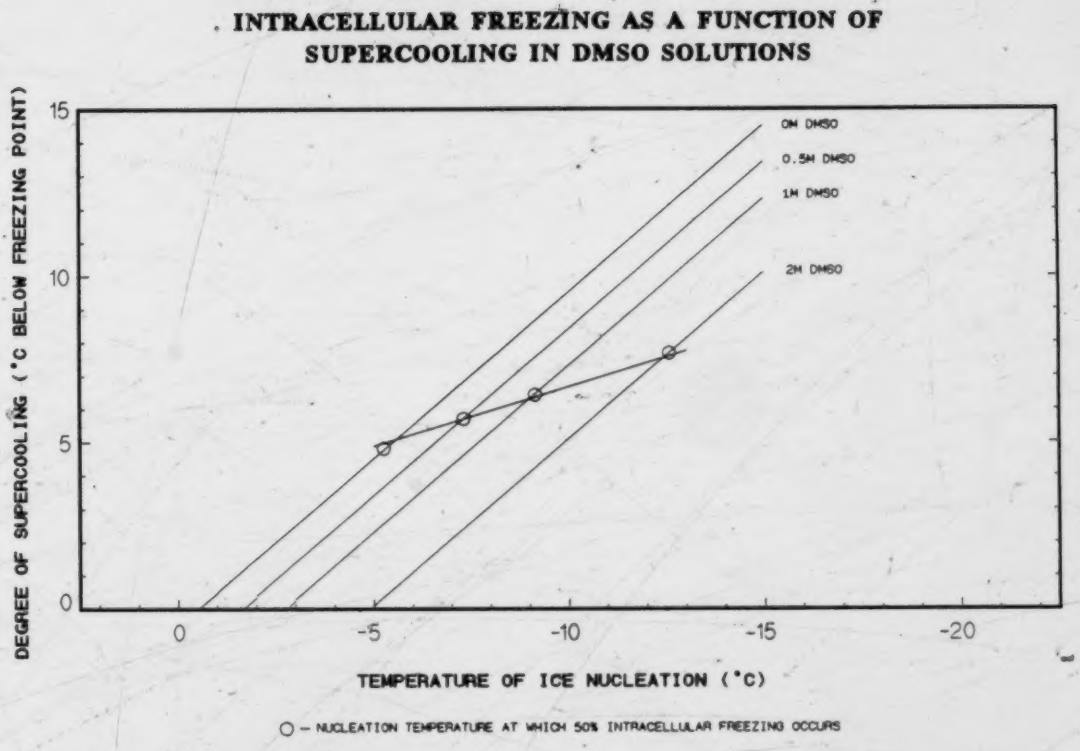

FIGURE 5.5

Graph showing the relationship between the degree of supercooling and the temperature of ice nucleation (essentially the same but offset so that the curves intersect the abscissa at the melting point) for four concentrations of DMSO. The temperatures at which 50% intracellular freezing occur are mapped onto the curves and a regression line is fitted for these points.

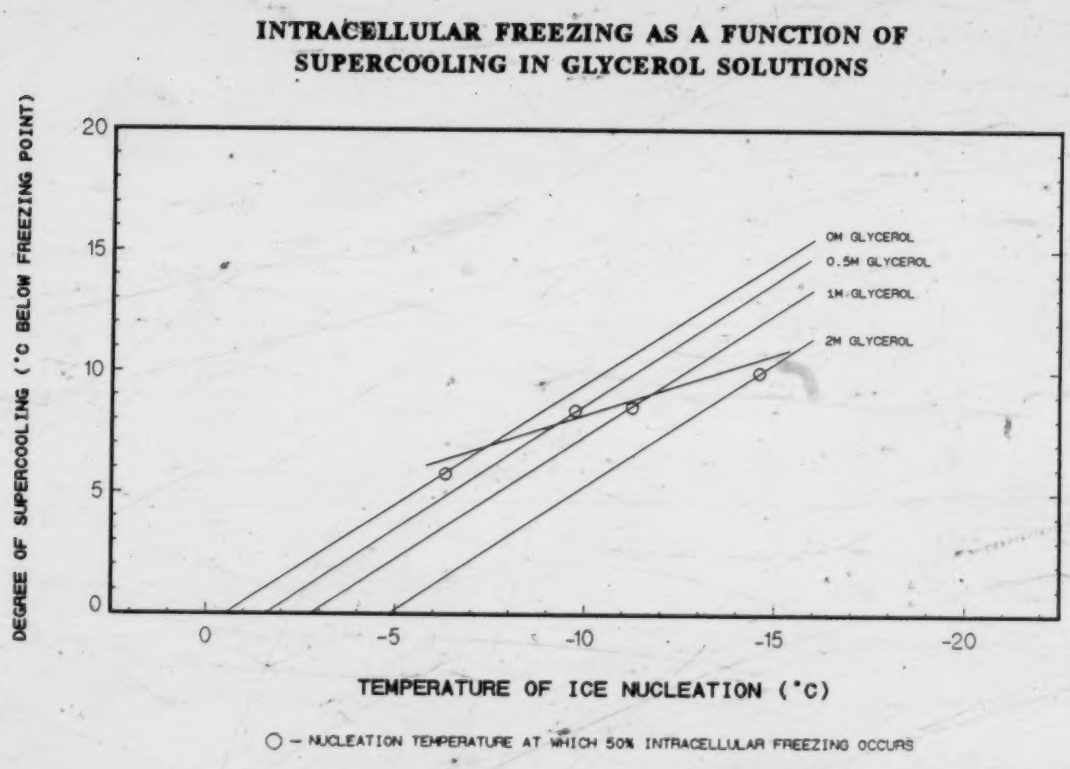

FIGURE 5.6

Graph showing the relationship between the degree of supercooling and the temperature of ice nucleation (essentially the same but offset so that the curves intersect the abscissa at the melting point) for four concentrations of glycerol. The temperatures at which 50% intracellular freezing occur are mapped onto the curves and a regression line is fitted for these points.

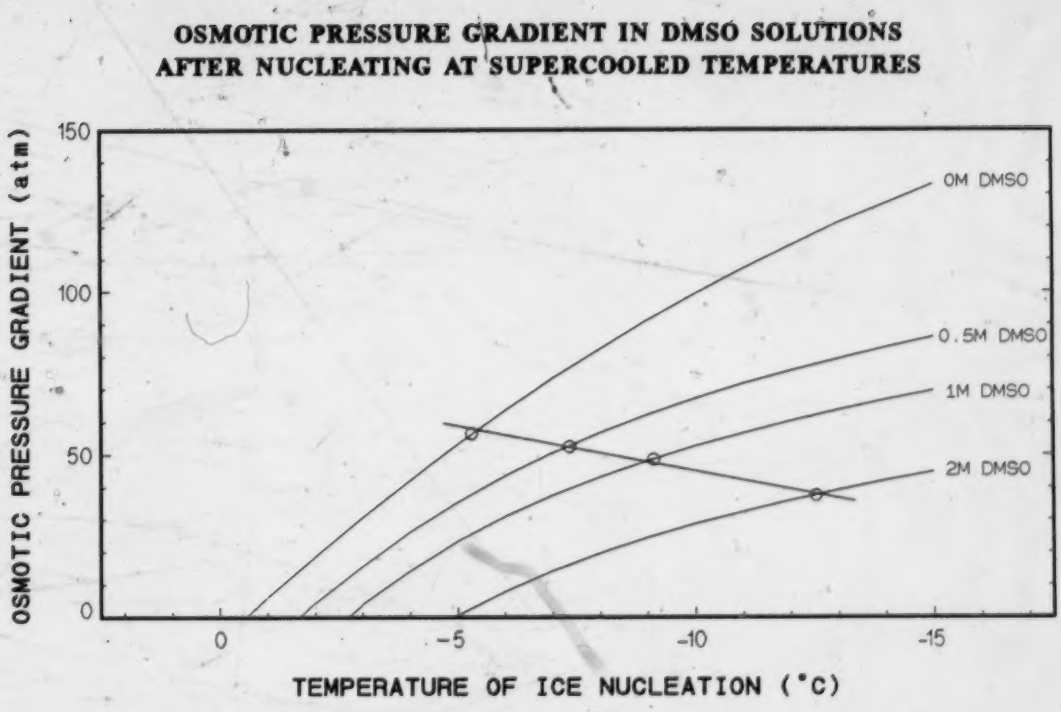

FIGURE 5.7

Graph showing the osmotic pressure gradient which develops across the cell membrane when ice is nucleated at supercooled temperatures in four concentrations of DMSO. The temperatures at which 50% intracellular freezing occur are mapped onto the respective curves and a regression line is fit for these four points.

OSMOTIC PRESSURE GRADIENT IN GLYCEROL SOLUTIONS AFTER NUCLEATING AT SUPERCOOLED TEMPERATURES

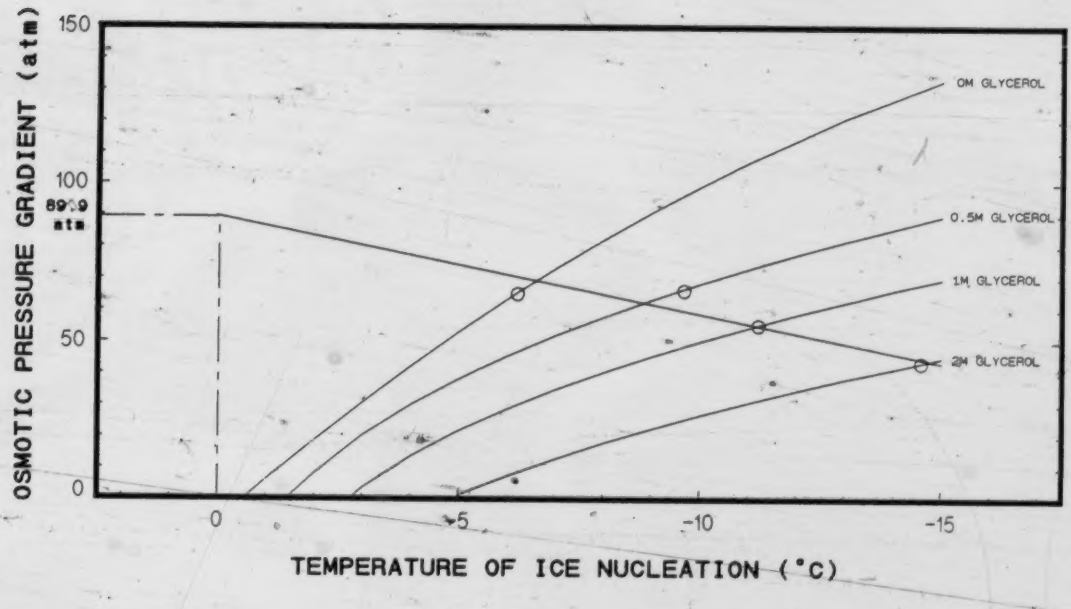

FIGURE 5.8

Graph showing the osmotic pressure gradient which develops across the cell membrane when ice is nucleated at supercooled temperatures in four concentrations of glycerol. The temperatures at which 50% intracellular freezing occur are mapped onto the respective curves and a regression line is fit for these four points and extrapolated to 0°C to show what osmotic pressure gradient would accompany 50% intracellular freezing at that temperature (89.9 atm).

cultures used for the two sets of data (a comparison of the two points at which 50% intracellular freezing occurs on the 1x MEM cases show that this is true). In figure 5.8, the line is extrapolated to 0°C where it intersects at an osmotic pressure gradient of 88.9 atmospheres.

5.4 Discussion

By plotting the percentage of intracellular freezing as a function of the temperature at which ice was nucleated, the nucleation temperatures at which 50% intracellular freezing occurs were found for each of the four concentrations of DMSO and glycerol. These points were then used to find the relationships between intracellular freezing and the velocity of the ice interface, the degree of supercooling of the protoplasm and the osmotic pressure gradient across the plasma membrane. These relationships will be used to critically analyze two existing theories and one new theory of intracellular ice formation.

The relationship between the velocity of the ice interface and the point of 50% intracellular freezing will be used to investigate Steponkus' theory that electrical transients at the ice interface cause a rupture in the cell membrane which allows the protoplasm to be nucleated. The relationship between the degree of supercooling which accompanies 50% intracellular freezing will be used to evaluate Levitt's hypothesis that intracellular ice formation is a result of critical supercooling of the protoplasm and the relationship between the osmotic pressure gradient which correlates with 50% intracellular freezing will be used to assess the theory of intracellular freezing which is the central theme of this thesis.

This theory states that if the osmotic pressure gradient across the plasma membrane reaches a critical level, the membrane ruptures and allows ice from the extracellular compartment to nucleate the protoplasm. When ice is nucleated at a subzero temperature, much of the water in the solution is removed to form ice. This leaves a high concentration of solutes in the unfrozen fraction of the solution which puts an osmotic stress on the cell. The cell will lose water in an attempt to equilibrate the osmotic pressure of its protoplasm with that of the external environment, but the rate of water loss is limited by the plasma membrane's permeability to water - the hydraulic conductivity. Since the cell cannot instantly equilibrate its osmotic pressure with that of the surrounding solution, an osmotic pressure gradient is created across the plasma membrane. The hypothesis holds that the plasma membrane is only capable of withstanding an osmotic pressure gradient with a magnitude that is lower than some critical value, which, if reached, will cause the membrane to rupture.

The data do not support the theory of intracellular ice as a result of electrical transients because the velocity of ice interface required to produce similar amounts of intracellular freezing is very different for different concentrations of the solutions. In glycerol, the velocity required to produce 50% intracellular freezing in 0.5M is three times that required to give the same degree of intracellular freezing in 2M (figures 5.3 & 5.4). If this theory were true then the temperatures for 50% intracellular freezing should have mapped onto the velocity curves as horizontal lines whereas the experimental evidence shows that

they had negative slopes (figures 5.3 & 5.4). There may have been an effect on the magnitude of electrical transients by the different solutions, although this should have caused the regression line to have a positive slope since the effect of increasing the solute concentration is to reduce the magnitude of electrical transients. (35)

The data also do not support the theory of intracellular freezing as a result of critical supercooling of the protoplasm. This theory predicts the points for 50% intracellular freezing should also map onto the supercooling curves as horizontal lines. The results presented in figures 5.5 & 5.6 show that they have a positive slope. It seems unlikely that ice formation would be more probable at higher temperatures when the degree of supercooling is identical. If intracellular ice formation was due to spontaneous nucleation of the protoplasm, this event should have ocurred at identical degrees of supercooling in all of the solutions tested.

The experimental evidence presented in figures 5.7 and 5.8 support the theory of intracellular freezing as a result of a rupture in the plasma membrane due to the attainment of an osmotic pressure gradient greater than a critical magnitude. The regression lines from these graphs have a negative slope which is predicted by the theory. Since the membrane is being ruptured by a physical force which exceeds its tensile strength, the force required to accomplish this will be smaller at colder temperatures (the membrane will be more brittle at colder temperatures and thus more easily disrupted). Thus the negative slopes of the regression lines support the theory. This line was extrapolated

to 0°C for the glycerol data giving an osmotic pressure gradient of 88.9 atm that should cause 50% of the cells' membranes to rupture. This prediction will actually be tested in the series of experiments to be presented in chapter 7. This is possible because both series of experiments were carried out using cells from the same culture.

CHAPTER VI

CELL INJURY ASSOCIATED WITH INTRACELLULAR FREEZING

6.1 Introduction

This experiment was designed to test whether the plasma membrane was the immediate site of injury related to the formation of intracellular ice. The plasma membrane of a healthy cell is able to provide a barrier to ice in a solution which is just slightly below its freezing point. The ice moves around the cell and generates virtually no physical deformation to the cell. In contrast, cells which do not have a continuous plasma membrane allow ice to move right into the protoplasm. This fact was exploited in this experiment to allow the integrity of the plasma membrane to be assayed after cells had frozen intracellularly. As a control, cells which underwent identical conditions but did not freeze intracellularly were assayed as well.

6.2 Experimental Protocol

Cells suspended in medium without cryoprotectant were supercooled on the cryomicroscope to either -6°C or -7°C. In order to get a symmetrical analysis, it was desired that approximately half of the cells studied should freeze intracellularly and the other half should not. To get these proportions, the cells should have been cooled to -6.5°C before nucleating ice. Instead of this, however, half of the cells were cooled to -6°C and the other half were cooled to -7°C before ice nucleation. This resulted in the same proportion of cells which froze intracellularly and assuming that intracellular ice formed at -6°C is the same as intracellular ice formed at -7°C, this method of

achieving the 50% split is just as good. Immediately after ice formed, the sample was warmed at +20°C/Min to +2°C to melt the ice. Then the sample was immediately cooled at -20°C/Min to -2°C where ice formation was initiated again. Figure 6.1 shows a graph of the complete cooling and warming protocol. During analysis, the cells were divided into two groups: those that froze intracellularly on the first freeze and those that did not. Upon freezing the second time, the cells from each group were assayed for damage to the plasma membrane by observing whether the membrane provided a barrier to the advancing ice interface.

6.3 Results

The upper row in table 6.1 shows the cells from the first freeze divided into those that formed intracellular ice and those that did not. Below each group are the results of the assay for membrane integrity that accompanied the second freeze. The results show very clearly that the formation of intracellular ice is accompanied by loss of ability to block the growing ice crystals from entering the protoplasm. Cells which are subjected to identical conditions but do not freeze intracellularly retain their barrier properties.

6.4 Discussion

This experiment shows very clearly that intracellular freezing correlates well with damage to the cell membrane. Since the cells in this experiment were observed during the approximately 30 seconds in which intracellular ice existed, it is an excellent chance to make observations of the morphological effects that ice inside the cell might have. Observation of the cells from the time intracellular ice formed

THERMAL PROTOCOL OF THE EXPERIMENT TO ASSESS MEMBRANE DAMAGE AFTER INTRACELLULAR FREEZING

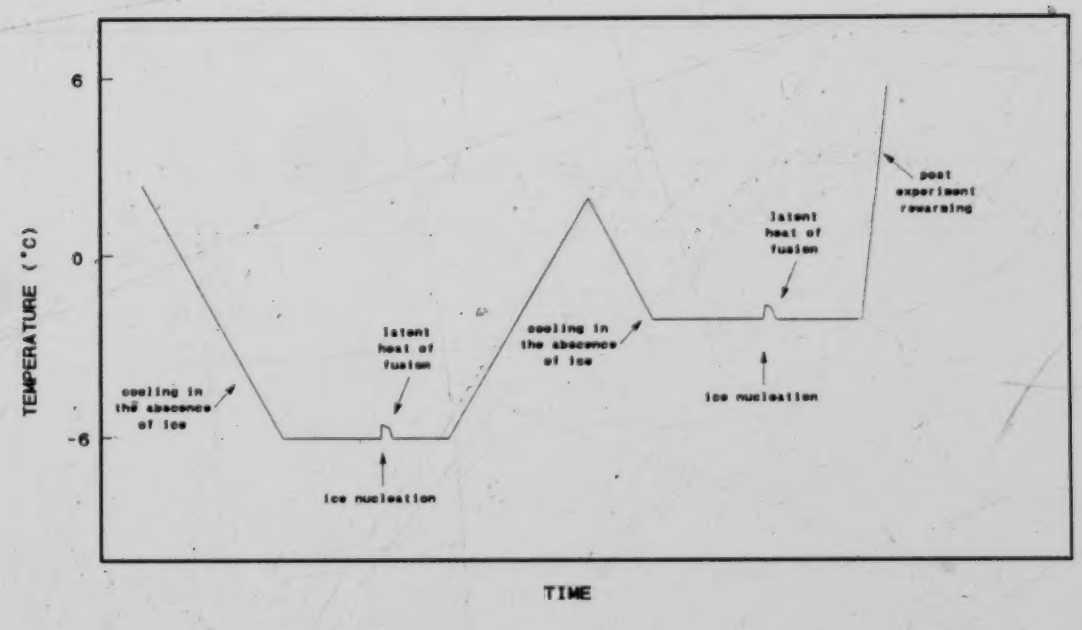

FIGURE 6.1

Shows the time-temperature curve followed by cells in the re-freezing experiments. The cells are cooled at -20°C/min to -6°C where ice is nucleated. The cells are then warmed at 20°C/min to 2°C to melt the ice. They are then re-cooled to -2°C where ice is nucleated again.

CORRELATION OF DAMAGE TO THE CELL MEMBRANE WITH INTRACELLULAR FREEZING

	NO INTRACELLULAR ICE		INTRACELLULAR ICE	
FIRST FREEZE	76		70	
	BARRIER	NO BARRIER	BARRIER	NO BARRIER
SECOND FREEZE	71	5	4	66

TABLE 6.1

Data showing whether the formation of intracellular ice is accompanied by damage to the cell membrane. The first freeze is to produce intracellular ice in about 50% of the cells and the second freeze is to assay which cells are capable of providing a barrier to an advancing ice front.

to the time that it melted gave no indication that physical forces were applied to the cell membrane by the formation or recrystallization of intracellular or extracellular ice. Despite this, the cells suffered virtually immediate damage to their membranes. This fact supports the concept that the intracellular ice was a result of a rupture in the cell membrane rather than the cause.

Both Steponkus' theory that intracellular ice forms as a result of electrical transients at the ice interface and the theory presented here that intracellular ice is a result of an excessive osmotic pressure gradient stipulate that the formation of intracellular ice is a manifestation of a rupture in the plasma membrane. The results of these experiments support this view as damage to the plasma membrane is highly correlated with the formation of intracellular ice. What of the cells which froze intracellularly but retained the barrier properties of their membranes? It is conceivable that these membranes were not too severely damaged by the rupture and were able to re-seal their membranes before the second freeze.

Mazur's theory that the damage associated with intracellular freezing is a result of recrystallization of the intracellular ice is not supported by these results. If the intracellular ice formed as a result of critical supercooling of the protoplasm (Levitt's hypothesis) then it would not be continuous with the extracellular ice - there would be no ice bridges extending through the plasma membrane. Thus recrystallization would have the effect of disrupting the intracellular components, not the membrane. If it did provide a physical force on the

membrane, then it is likely that this would be observable. If Mazur's hypothesis of the formation of intracellular ice being due to ice crystals propagating through aqueous pores in the plasma membrane is true, then recrystallization could very well disrupt the membrane without being observed morphologically.

CHAPTER VII

OSMOTIC PRESSURE GRADIENT AS A CAUSE OF DAMAGE TO CELLS IN THE ABSENCE OF ICE

7.1 Introduction

The hypothesis emerging from this work is that the plasma membrane of a cell will rupture when the gradient in osmotic pressure across the membrane exceeds a critical value. If this occurs during freezing, then intracellular freezing results as ice from the extracellular compartment will nucleate the protoplasm. This set of experiments was designed to test whether a high osmotic pressure gradient in the absence of ice (at 0°C) could cause damage to the cell membrane. If the above mentioned hypothesis is correct, it should be possible to demonstrate that an osmotic pressure gradient of a certain magnitude is sufficient to cause a rupture in the plasma membrane. In order to do this, the osmotic pressure gradient must occur in a non-freezing environment since it is impossible to control the many confounding factors which accompany the formation of an osmotic pressure gradient during freezing. Once this is demonstrated, the magnitude of the osmotic pressure gradient that ruptures 50% of the cells can be compared with the value that was extrapolated to 0°C in the supercooling experiments (see figure 5.8).

7.2 Experimental Protocol

Cells were held at 0°C and DMSO was added to bring the concentration up to 3M, 4M or 5M. Two conditions were used for each of these concentrations: slow addition and rapid addition. In the slow addition group, the DMSO was added stepwise allowing the osmotic pressure to equilibrate between additions. This insured that the

maximum osmotic pressure gradient that the cells experienced was very small. In the rapid addition group, the DMSO was added all at once so that the cells would experience the maximum osmotic pressure gradient (the assumption of ideal mixing was made thus the osmotic pressure gradient is easily calculated from the number of moles of DMSO added). After the final concentration was reached, each group was allowed to stand for two minutes. The two groups were then diluted slowly with tissue culture media; all groups were diluted with the identical protocol as defined in the chapter on Materials and Methods (chapter III). After dilution, the cells were centrifuged at 500 g for 45s and all but 0.5 ml of the supernatant was removed. The cells were then resuspended by agitation and assayed for membrane integrity by testing whether they provided a barrier to ice formation on the cryomicroscope. Figure 7.1 illustrates this protocol diagramatically.

7.3 Results

Table 7.1 shows the original data from the experiment. In the slow addition group, there was virtually no indication of damage to the cell membranes at any concentration of DMSO. In the rapid addition group, however, damage increased with increasing concentrations of DMSO. The data are plotted as a function of osmotic pressure gradient in figure 7.2. A linear regression fit is plotted which yields an r value of 0.996 for the rapid addition group and 0.998 for the slow addition group. The osmotic pressure gradient at which 50% of the cells are damaged is interpolated from the graph as 90.3 atm.

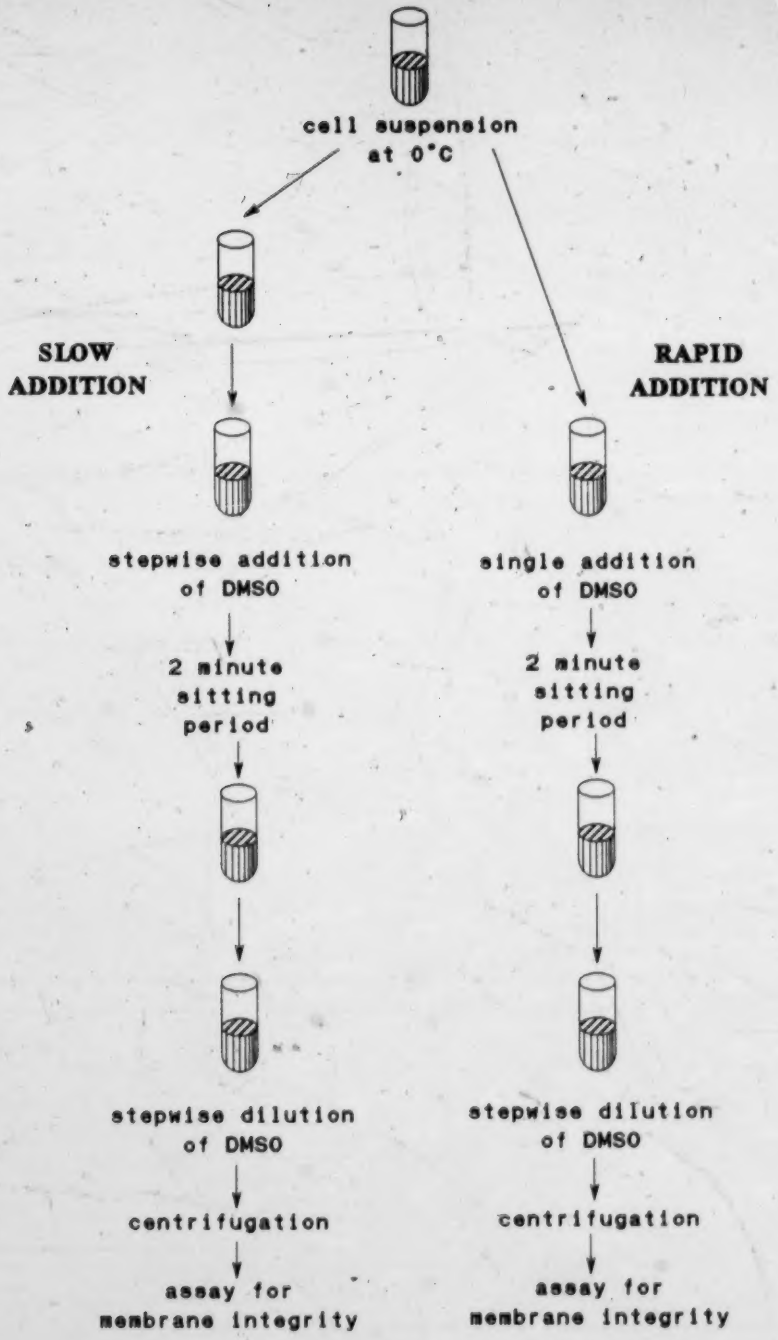

FIGURE 7.1

Protocol for the treatment of cells for rapid and slow addition of DMSO. The protocols are identical after the DMSO has been added.

OSMOTIC PRESSURE GRADIENT AT 0°C

	RAPID ADDITION		SLOW ADDITION	
	BARRIER	NO BARRIER	BARRIER	NO BARRIER
3M DMSO	56	7	47	1
4M DMSO	32	28	48	0
5M DMSO	16	44	57	1

TABLE 7.1

Table showing the relative numbers of cells which can and can not provide a barrier to an advancing ice front after rapid or slow addition of DMSO to reach one of the three concentrations indicated.

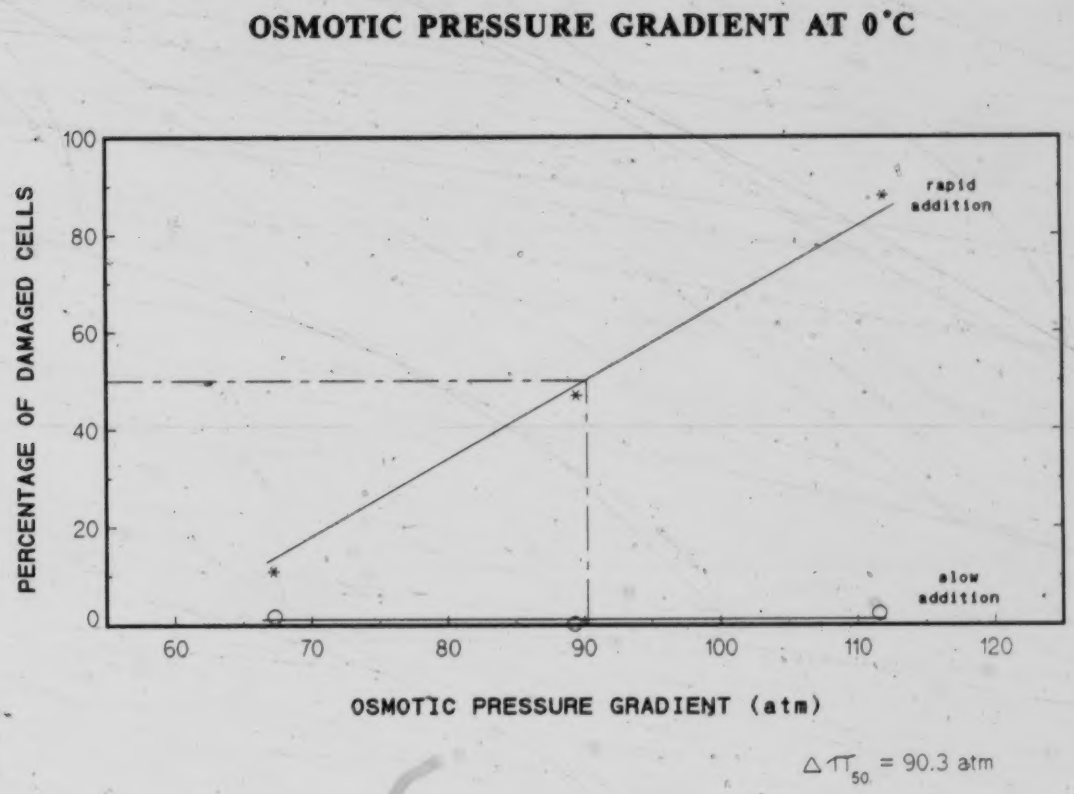

FIGURE 7.2

Graph showing the osmotic pressure gradient produced as a result of rapid addition of DMSO and the proportion of cells which were damaged by the rapid addition. The osmotic pressure gradient that corresponds to 50% damage is indicated.

7.4 Discussion

The data show clearly that a high osmotic pressure gradient can be responsible for damage to cells. The only differences between the two groups was the kinetics of addition of DMSO and the length of time that each group was exposed to DMSO. The slow addition group was exposed to DMSO much longer than the rapid addition group and could therefore have been affected by DMSO toxicity. The results show almost no indication of a loss of the barrier properties of the membrane to ice in the slow addition group thus any toxicity effects that the DMSO may have had did not show up in this experiment. The vast difference between the barrier properties of cells in the rapid addition group and those of cells in the slow addition group can only be attributed to the kinetics of addition of DMSO and therefore to the magnitude of osmotic pressure gradient that existed across the plasma membrane during the addition step.

Since the cells used in this experiment were from the same culture as those used in the glycerol supercooling experiment, the data from this experiment can be compared to the extrapolation to 0°C from the supercooling experiments where the value of the critical osmotic pressure gradient (that which produced 50% intracellular freezing) for that temperature was 89.9 atm (figure 5.8). This was the osmotic pressure gradient which should have caused 50% damage to the cells at 0°C had it been possible to conduct the experiment at that temperature. The osmotic pressure gradient which caused 50% damage at 0°C in this experiment was measured to be 90.3 atm. The critical osmotic pressure gradient was determined for this cell culture using two very different

experimental approaches. The fact that the values that resulted came out to be almost identical lends additional credence to the hypothesis that plasma membranes are ruptured by a critical osmotic pressure gradient during rapid cooling.

The critical magnitude of osmotic pressure gradient will be specific for a certain cell type. This is because of the differences that exist in the plasma membranes of different cells: the permeability properties, the tensile strength, the composition and other properties of membranes will determine how large an osmotic pressure gradient is required to cause a rupture in a membrane. For this reason, the data obtained in the supercooling experiments in the presence of DMSO (see figure 5.7) were not compared to the results of this experiment; the two experiments used different cell cultures which obviously had different hydraulic conductivities. The supercooling experiment in the presence of glycerol used cells from the same culture as those used in this experiment therefore the comparison was meaningful.

CHAPTER VIII

DISCUSSION

8.1 Re-assessment of Current Theories of Intracellular Freezing

In light of the data presented in chapters three through seven, a critical re-assessment of the mechanism of intracellular ice formation is needed. The three prevailing theories on the genesis of intracellular ice have not been supported by the experiments carried out in this study, therefore, it is necessary to propose a new theory which is consistent with the data. This proposed theory is that intracellular freezing is a result of a rupture in the plasma membrane which in turn is caused by the gradient in osmotic pressure that can exist across the cell membrane during rapid cooling. The gradient is created as a result of the cell's inability to lose water fast enough to maintain osmotic equilibrium with the extracellular environment. Before discussing this theory in more detail and the evidence that supports it, a review of the data in relation to the three previous theories will be presented.

8.1 (a) Intracellular Freezing as a Result of Critical Supercooling

Levitt's hypothesis that intracellular freezing is a result of excessive supercooling of the protoplasm is not consistent with the data presented in chapter 5. The theory states that when cells are cooled very rapidly, the protoplasm becomes increasingly supercooled if osmotic equilibrium is not maintained. Because the solute concentration in the protoplasm is much lower than in the extracellular unfrozen solution, it exists in solution at a temperature below its freezing point: this is the supercooled state. If the protoplasm should become supercooled to a

critical degree, then nucleation becomes likely and intracellular freezing will occur.

The plots of degree of supercooling vs. the temperature of ice nucleation (figures 5.5 and 5.6) should have yielded horizontal lines when the points at which 50% intracellular freezing occurred were plotted on the graph. This is because the same amount of intracellular freezing should have occurred when the identical degree of supercooling was produced. In this theory, it is the attainment of a critical degree of supercooling which caused intracellular freezing; the amount of supercooling required to cause 50% of the cells to freeze internally should therefore have been the same for each solution. Instead, a line with a positive slope was the result. This implies that the formation of intracellular ice would have to be a temperature dependent function with intracellular freezing occurring more readily at higher temperatures (assuming equal degrees of supercooling). There is no basis in the hypothesis which allows these results to be rationalized therefore the theory must be rejected.

8.1 (b) Intracellular Freezing as a Result of Ice Passing Through Aqueous Membrane Pores

Mazur proposed that intracellular ice forms as a result of extracellular ice crystals propagating through the aqueous pores in the cell membrane. The conditions under which freezing is conducted (cooling rate, composition of solution, temperature) affect the geometry of the advancing ice crystals. If the conditions are such that the minimum radius of growing ice crystals is the same as the radius of aqueous pores in the plasma membrane, then the ice crystals can

propagate through these pores thereby nucleating the protoplasm. This theory is not supported by the evidence presented in chapter four. When a cell suspension is cooled at a constant rate, the temperature at which intracellular ice forms would be predicted by this theory to be dependent on the rate at which cooling is taking place. This is because the size and shape of the ice crystals at a given temperature will be dependent on the cooling rate. Assuming that the radius of the aqueous pores remains relatively constant, intracellular freezing should result when the minimum radius of advancing ice crystals reaches a critical value. The data in chapter 4 shows that this is not the case: the temperature at which intracellular ice forms is constant, regardless of the rate of cooling. The minimum radius of ice crystals in the extracellular environment will be much different at this temperature depending on the cooling rate. This shows that the geometry of the ice crystals at the ice-liquid interface is not a critical factor in the formation of intracellular ice.

8.1 (c) Intracellular Freezing as a Result of Electrical Transients at the Ice Interface

Steponkus' theory of the formation of intracellular ice asserts that electrical transients occur at an advancing ice interface as a result of the selective exclusion of one sign of charge from the ice interface. These electrical transients will cause the plasma membrane to rupture if they reach a critical magnitude, allowing extracellular ice to nucleate the protoplasm (35). That intracellular freezing is a result of a rupture in the plasma membrane is consistent with the data presented in chapter six but the theory that this rupture is a result of

electrical transients at the ice interface is not supported by the evidence from chapters four and five. The magnitude of the electrical potential difference that exists between the ice phase and liquid phase at the interface is proportional to the velocity of the interface (35). The data from chapter four show that when cooling at a constant rate, intracellular freezing occurs at the same temperature regardless of what that cooling rate is. The velocity of the ice interface, at a given temperature, is dependent on the cooling rate (for a specific solution). Thus, intracellular freezing occurs under conditions of different ice interface velocities, contradicting the theory. The evidence from chapter five also conflicts with the theory on the grounds of the magnitude of the ice interface velocity (which was measured directly in these experiments) as it corresponds to the observed formation of intracellular ice. Different ice interface velocities were required to produce similar amounts of intracellular freezing in the various solutions. It is true that the composition of the solution could play a role in determining the magnitude of the electrical transients which occur at the ice interface, however, the nature of this dependence is thus: dilute aqueous solutions produce the greatest electrical transients. High concentrations of solute reduce the effect due to the turbulent nature of solution at the ice-solution interface (35). The data from chapter five show that lower velocities are required with increasing solute concentration to achieve similar amounts of intracellular freezing. This is the opposite of what would be expected if the theory of electrical transients as a cause of intracellular freezing was true.

Thus, the three existing hypotheses of the formation of intracellular ice are not supported by the evidence and must be supplanted with a new theory. This thesis presents the theory that intracellular ice is a result of a rupture of the plasma membrane which is caused by the attainment of a critical osmotic pressure gradient across the membrane. Before examining this theory in relation to the data presented in this work, the nature of the damage which accompanies intracellular freezing will be analyzed with respect to existing theories.

8.2 Nature of Injury Which Accompanies Intracellular Freezing

8.2 (a) Recrystallization of Intracellular Ice

Mazur's theory states that it is the recrystallization, not the actual formation, of intracellular ice which causes damage and targets the protoplasm as the site for injury (18). If the intracellular ice is not continuous with the extracellular ice, then recrystallization of intracellular ice will very likely not affect the plasma membrane (morphological observations show no indication of physical deformations of the membrane during recrystallization of intracellular ice). The evidence from this study, however, shows that the isolation of intracellular ice from the extracellular ice is not probable (only Levitt's theory of critical supercooling of the protoplasm could lead to this situation). With ice bridges passing through the plasma membrane connecting the intracellular and extracellular compartments, recrystallization could ostensibly enlarge these bridges leading to the formation of even larger disruptions of the membrane. Mazur's theory

that intracellular ice forms as a result of extracellular ice crystals passing through aqueous pores in the membrane is the only theory in which recrystallization could actually create a hole in the membrane. As the ice in the aqueous pore recrystallizes, it will expand the pore to a size that is much greater than can be resealed upon thawing. As was discussed above, however, this theory is not very likely, thus the implication of recrystallization as a cause of damage associated with intracellular freezing is fortuitous.

8.2 (b) Intracellular Ice as a Result of a Rupture in the Plasma Membrane

The only competing theory is that intracellular ice formation is a result of damage to the cell membrane. A rupture in the membrane allows extracellular ice to pass through the membrane and nucleate the supercooled intracellular compartment. This theory is consistent with all the data presented in this study. The theory predicts that the vast majority of cells which have frozen intracellularly will not be able to present a barrier to an advancing ice front as was shown in chapter six. This theory is incorporated into the hypothesis being presented in which the osmotic pressure gradient is viewed as the cause of intracellular freezing.

8.3 Prevention of Intracellular Ice

Presently, there are two methods for preventing intracellular ice from forming in cells during rapid cooling. The first is a two step cooling process in which the cells are cooled (after ice nucleation just below the freezing point) to a temperature above that at which

intracellular ice is likely to form and held there until osmotic equilibrium occurs. The cells are then cooled rapidly down to the storage temperature. The second method involves the use of non-penetrating cryoprotectants. These are generally long chain polymer molecules which have very high osmotic coefficients (they reduce the osmotic pressure in a solution to a much greater extent than would be predicted on the basis of their molar concentration in the solution). These compounds have the effect of causing the cells to shrink extensively before freezing (in order to obtain osmotic equilibrium with the solution). The large size of these polymers makes them relatively immobile in an ice matrix thus preventing them from concentrating in the unfrozen fraction. The common thread between these two techniques lies in the fact that both methods concentrate the protoplasm before rapid cooling. The fact that this can allow the cell to avoid intracellular ice formation must be explained by any theory which attempts to elucidate the nature of intracellular freezing.

8.4 Critical Osmotic Pressure Gradient as a Cause of Intracellular Ice

The one theory which is supported by all the data in this study as well as being able to explain the strategies for avoidance of intracellular ice is the central hypothesis of this work: that intracellular freezing is a result of a rupture in the plasma membrane which is caused when a critical osmotic pressure gradient between the inside and outside of the cell is reached. This theory is supported by the experiments conducted in this study through both the targeting of the membrane as the site of damage and the physical events which can be correlated with intracellular ice formation. The two techniques for

avoiding intracellular freezing during rapid cooling are natural consequences of this theory as concentrating of the protoplasm before freezing reduces the osmotic pressure gradient that will occur during rapid cooling. The primary difficulty with this theory is interpreting how an osmotic pressure gradient can lead to a physically damaging force which can cause the rupturing of a cell membrane. The osmotic pressure of a solution is simply a property of the solution, an osmotic pressure gradient across a cell membrane is not the same thing as a hydrostatic pressure gradient across that membrane. It could lead to a hydrostatic pressure if the membrane was held in position but this does not happen; the membrane shrinks as water leaves the cell. If this is the case, then what force is present which actually breaks the membrane? The answer lies in the fact that, although there is no hydrostatic pressure, water moves out of the cell exactly as if there was such a pressure. This outward movement of water creates a drag on the membrane due to the friction between water and the pores which it travels through. The plasma membrane for any cell type has specific properties which determine how permeable it is to various substances. The permeability to water is known as the hydraulic conductivity of the membrane. When water moves through any channel, it encounters a resistive force due to friction on the sides of this channel; the aqueous pores in a cell membrane are no different. Thus the outward force due to friction could concievably rupture the membrane if the water flux reached a velocity at which the tensile strength of the membrane was exceeded by the frictional force.

The osmotic pressure gradient that the cells are faced with in the experiment described in chapter six can be readily estimated due to the availability of published equations which describe the behavior of the ternary systems used (25,26). By mapping the intracellular freezing data onto the osmotic pressure gradient curves, a line with a negative slope results. This implies that the dependence of intracellular ice formation on the critical osmotic pressure gradient is a temperature dependent function. This is completely compatible with the theory as the tensile strength of any material is temperature dependent. At lower temperatures the membrane becomes more brittle and can be ruptured with less force than at higher temperatures. In chapter seven, it was shown that a high osmotic pressure gradient can indeed cause damage to membranes in the absence of ice (both intracellular and extracellular). By comparing the osmotic pressure gradient required to damage 50% of the cells in this experiment (88.9 atm) with the extrapolated point at which 50% intracellular freezing would occur at 0°C from the experiment in chapter five (90.3 atm) an excellent agreement is found. The fact that the relationship between the critical osmotic pressure gradient and the formation of intracellular ice is temperature dependent and the excellent correlation when the function is extrapolated to 0°C provides strong evidence in favour of this theory.

8.5 Avoidance of Intracellular Freezing With Respect to the Osmotic Pressure Gradient

The only remaining question is how this theory predicts the techniques which are used to avoid intracellular freezing. Both strategies create shrunken cells before rapid cooling which concentrates

the solutes in the protoplasm. This serves to lessen the osmotic pressure gradient during rapid cooling because the protoplasm is already very concentrated when the rapid cooling is begun. Thus the osmotic pressure gradient can never develop to a magnitude that would lead to the rupturing of cell membranes and intracellular freezing does not occur.

There is another method for avoiding intracellular freezing that has not yet been dealt with: the technique of vitrification. This method avoids freezing altogether, both intracellular and extracellular. By making a very concentrated solution, ice formation can be avoided during cooling and the sample can become an amorphous solid (a glass). This procedure is currently being used to preserve cell suspensions and small tissues and is being applied in research projects aimed at preserving whole organs. Without extracellular ice, the only way for intracellular freezing to occur would be by critical supercooling of the protoplasm. This is highly unlikely, however, as the protoplasm is in osmotic equilibrium with the surrounding solution and will therefore form a glass as well.

8.6 Consequences of This Work

This study has provided a novel theory of the mechanism by which ice forms inside cells during rapid cooling. Naturally, there are several immediate implications which present themselves as a result of this theory. When cooling cells for preservation, it is valuable to be able to predict the conditions under which intracellular ice will form since it is desirable to avoid it. It is also desirable to cool cells

as rapidly as possible to limit the time spent in the high solute concentrations that are produced by freezing. Thus the protocol should be optimized to give the fastest rate of cooling possible without forming intracellular ice. According to the osmotic pressure gradient theory, if the hydraulic conductivity of the plasma membrane (its permeability to water) is known along with the membrane's tensile strength, then it is possible to predict the maximum osmotic pressure gradient that the cell will be able to accommodate without rupturing. These two properties may well be closely related as they both describe the microstructure of the plasma membrane. Thus it may be possible to predict intracellular freezing based solely on the hydraulic conductivity of the plasma membrane. This would be desirable as this property has been measured for a variety of cell types and can be readily measured (19,20).

The next step to be taken with this work is to apply it to multicellular systems. The effects of ice in the intercellular space must be studied as well as the possibility of manipulating the osmotic environment at various levels within a tissue. This work should be pursued with gusto as the implications of this thesis provide a very optimistic view that the current problems impeding the development of techniques for preserving organs at low temperatures can be overcome making the banking of organs and tissues a reality.

CHAPTER 9

CONCLUSION

To conclude this thesis, it will be valuable to review the objectives that were set out at the beginning. The questions posed were: how does intracellular ice form during rapid cooling and what is the nature of damage that accompanies its formation. It was shown that previous theories could not fully explain the experimental data that was generated by this study and therefore a new hypothesis was proposed: that the osmotic pressure gradient that develops across a cell membrane when cooling is too rapid to allow the cell to remain in osmotic equilibrium with the extracellular environment can cause a rupture in the cell membrane which allows extracellular ice to nucleate the protoplasm. This theory is consistent with the data and allows other observations to be explained. For instance, if cells are partially dehydrated before rapid cooling is initiated, then intracellular ice will not form. Within the framework of the proposed theory, this can be explained by the fact that the initial dehydration lessens the osmotic pressure gradient that forms during rapid cooling. Thus it is reasonable to consider this theory as the most useful basis for analyzing any event in which biological materials are rapidly frozen.

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APPENDIX A

EQUATIONS DESCRIBING THE TERNARY SYSTEMS: DMSO-NaCl-WATER AND GLYCEROL-NaCl-WATER

Three previously published equations were used to calculate both the melting points and the solute concentrations of the various solutions of DMSO and glycerol. For the DMSO solutions, one equation was suitable for calculating both the melting point and the composition of the solution at a given temperature. (26) Refering to equation 1, S is set to the initial concentration of solutes and solving for Tm gives the melting point of the solution. To calculate the solute concentration at a given temperature, let Tm be the temperature and solve for S.

$$Tm = \left[-0.6 + 0.17 \tan^{-1} R \right] S + \left[\frac{\tan^{-1}(R/2)}{132} - 0.001 \right] S^2 - 0.00045 S^3 \qquad \text{(eq. 1)}$$

Tm = melting temperature of solution (°C)
S = concentration of total solutes (g/100g)
R = weight ratio of DMSO/NaCl

Obtaining the melting points for the glycerol solutions was not as simple since Pegg's equation (25) did not provide satisfactory results for the solutions being used in this study (the equation was not derived from data with such a low concentration of total solutes for a given R value). Thus the melting points were obtained using Fahy's simplification (6) of the thermodynamic approximation of the melting point (equation 2).

$$\frac{610.75\ (273.15 - Tm)}{Tm\ (273.15)} + 4.882\ \ln\left[\frac{Tm}{273.15}\right] = \ln\ (1 - Xs) \quad \text{(eq. 2)}$$

Tm = melting temperature of solution (°K)
Xs = solute mole fraction

Pegg's equation (25) was used to calculate the composition of the solution for a given temperature, however, to allow for the error in melting points, a constant was added to the temperature for each solution. Refering to equation 3, the difference between the melting point calculated from equation 2 and that calculated from equation 3 was added to Tm when solving for S in equation 3.

$$Tm = S\ (-1.6 - 1.27R - 0.25R^{2})^{-1} - 0.01S^{2} \quad \text{(eq. 3)}$$

Tm = melting temperature of solution (°C)
S = concentration of total solutes (g/100g)
R = weight ratio of glycerol/NaCl